

Interaction of Internal Anions with Potassium Channels of the Squid Giant Axon

D. J. ADAMS and G. S. OXFORD

From the Department of Physiology, University of North Carolina, Chapel Hill, North Carolina 27514; and The Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT The interaction of internal anions with the delayed rectifier potassium channel was studied in perfused squid axons. Changing the internal potassium salt from K^+ glutamate $^-$ to KF produced a reversible decline of outward K currents and a marked slowing of the activation of K channels at all voltages. Fluoride ions exert a differential effect upon K channel gating kinetics whereby activation of I_K during depolarizing steps is slowed dramatically, but the rate of closing after the step is not much altered. These effects develop with a slow time course (30–60 min) and are specific for K channels over Na channels. Both the amplitude and activation rate of I_K were restored within seconds upon return to internal glutamate solutions. The fluoride effect is independent of the external K^+ concentration and test membrane potential, and does not recover with repetitive application of depolarizing voltage steps. Of 11 different anions tested, all inorganic species induced similar decreases and slowing of I_K , while K currents were maintained during extended perfusion with several organic anions. Anions do not alter the reversal potential or shape of the instantaneous current-voltage relation of open K channels. The effect of prolonged exposure to internal fluoride could be partially reversed by the addition of cationic K channel blocking agents such as TEA $^+$, 4-AP $^+$, and Cs $^+$. The competitive antagonism between inorganic anions and internal cationic K channel blockers suggests that they may interact at a related site(s). These results indicate that inorganic anions modify part of the K channel gating mechanism (activation) at a locus near the inner channel surface.

INTRODUCTION

Control of the ionic composition of the intracellular milieu of nerve and muscle cells by internal perfusion serves as the basis for many important studies of voltage-dependent ionic channel function in excitable membranes. The ability to systematically alter the intracellular ionic environment as well as to introduce pharmacological agents directly onto the inner membrane surface has become an indispensable tool. With the advent of such techniques it soon became apparent that the functional properties of the sodium and

Address reprint requests to Dr. G. S. Oxford, Dept. of Physiology, University of North Carolina, Medical Research Wing 206H, Chapel Hill, NC 27514. Dr. Adams' present address is Dept. of Pharmacology, University College, London, England.

potassium channels of nerve membrane could be affected by the ionic composition of the internal perfusion solution (e.g., Baker et al., 1962; Adelman et al., 1966; Chandler and Meves, 1970). Recently, studies have centered on the effects of internal monovalent cations on the selectivity properties (Cahalan and Begeenich, 1976; Ebert and Goldman, 1976; Begeenich and Cahalan, 1980) and the gating behavior (Schauf and Bullock, 1978; Oxford and Yeh, 1979; Horn et al., 1981) of sodium channels, although similar work on voltage-dependent potassium channels has not yet appeared.

The importance of possible ionic influences upon voltage-dependent potassium channel gating is highlighted by the recent measurements of single potassium channel currents in the squid giant axon (Conti and Neher, 1980). In these experiments the axon membrane was exposed to solutions that were unphysiological and asymmetrical with respect to the normal potassium concentrations. Interpretation of such single-channel measurements for proposing new models of the gating process may be difficult without independent knowledge of the influences of cations and anions on channel function.

In this paper we show that the anion composition of the internal perfusion solution of squid giant axons has profound effects upon the amplitude and kinetics of potassium channel gating and that the site of action of these anions is related to those previously postulated to be involved in the action of several cationic blocking agents. A preliminary report of this work has appeared (Adams and Oxford, 1981).

METHODS

Experiments were performed on single giant axons isolated from *Loligo pealei* at the Marine Biological Laboratory, Woods Hole, MA. Axon diameters ranged from 350 to 500 μm (mean = 430, $n = 28$). Axons were cleaned of most adhering tissue, and the axoplasm was squeezed out with a tiny rubber roller and replaced with an artificial internal solution introduced via a micrometer syringe. Axons were then mounted in a Plexiglas chamber, continuously perfused both internally ($\sim 80 \mu\text{l}/\text{min}$) and externally, and voltage-clamped with conventional axial wire techniques. Details of the electronics and procedures of voltage-clamping have been previously published (Oxford et al., 1978; Oxford, 1981). All experiments employed analog electronic subtraction of much of the leakage and capacitative currents.

Most, and on several occasions all, of the measured resistance in series with the axon membrane was continuously compensated by positive electronic feedback (Oxford, 1981). Adjustment of series resistance compensation was performed under voltage clamp at the beginning of each experiment in a control artificial seawater solution by the following procedure. The membrane was step-depolarized in 1-mV increments near the potential level where maximum inward sodium current was observed. Once the potential corresponding to this peak had been noted, a depolarizing prepulse was applied that inactivated at least 80% of the sodium conductance. Again the potential of maximum inward current was located to within 1 mV. The discrepancy between the two values of membrane potential was assumed to be due only to the voltage drop across the series resistance and was nulled with the feedback potentiometer such that a determination of the voltage for peak sodium current with

and without a prepulse yielded the same or nearly the same value. This procedure was adopted following the suggestion of Drs. J. Starkus (University of Hawaii) and T. Begeisich (University of Rochester), and was in practice a very convenient and more consistent method of determining the degree of compensation required than was the conventional current step procedure (see Binstock et al., 1975).

The axons were bathed in an artificial seawater solution of the following composition: 445 mM NaCl, 10 mM KCl, 50 mM CaCl₂, and 5 mM HEPES buffer adjusted to a final pH of 7.8. In several experiments a high external potassium seawater was used to reduce problems associated with potassium ion accumulation in the periaxonal space during long depolarizations. The high-K solution (HKS_W) contained 220 mM

TABLE I
Composition of Internal Solutions

Solution*	Anion	Concentrations			Sucrose
		Na	K	Phosphate buffer [‡]	
		<i>mM</i>	<i>mM</i>	<i>mM</i>	
SIS	320 glutamate 50 fluoride	50	350	15	300
320 NaGlu	320 glutamate	320	0	15	370
320 KGlu	320 glutamate	0	320	15	370
320 KAsp	320 aspartate	0	320	15	370
320 KF	320 fluoride	0	320	15	370
320 KCl	320 chloride	0	320	15	370
320 KBr	320 bromide	0	320	15	370
320 KNO ₃	320 nitrate	0	320	15	370
160 K ₂ PO ₄	160 phosphate	0	320	15	470
160 K ₂ SO ₄	160 sulfate	0	320	15	450
320 KIs	320 isethionate	0	320	15	370
107 K ₃ Cit	107 citrate	0	320	15	520
506 KMOPS	506 MOPS [‡]	0	506	15	250

* The final pH was adjusted to 7.3 with anion acid and the osmolarity of internal solutions was adjusted to ~1,100 mosmol.

[‡] Phosphate buffer: KH₂PO₄ titrated with KOH to pH 7.3.

[‡] MOPS: 3-(*N*-morpholino) propane sulphonic acid; pK_a 7.2.

KCl, 225 mM *N*-methylglucamine (NMG) Cl, 50 mM CaCl₂, and 5 mM HEPES buffer. Unless otherwise indicated in the figure legends, all external experimental solutions contained 300 nM tetrodotoxin (TTX; Sigma Chemical Co., St. Louis, MO) to block ionic current through sodium channels.

The compositions of the standard internal solution (SIS) and all internal experimental solutions are given in Table I. It is important to note that the experimental solutions contained no sodium. This precaution was taken to avoid the well-known blocking action of internal sodium at positive membrane potentials (Bergman, 1970; Bezanilla and Armstrong, 1972; French and Wells, 1977). Also note that all internal solutions contained 15 mM phosphate (HPO₄) buffer to adjust the internal pH to 7.3. This amount of internal phosphate had no significant effect upon our results as it accounted for only 7% of the total concentration of internal anions. This is

equivalent to the HPO_4 concentration occurring naturally in *Loligo pealei* axoplasm (Deffner, 1961). The osmolarity of the solutions was adjusted to $\sim 1,100$ mosmol by the addition of sucrose. In some experiments tetraethylammonium bromide (TEA; Eastman Kodak Co., Rochester, NY), 4-aminopyridine (4-AP; Sigma Chemical Co.), cesium fluoride (MCB Chemicals, Gibbstown, NJ, or Alfa Chemicals, Danvers, MA), and EDTA (dipotassium salt) (Sigma Chemical Co.) were used in the internal medium. Details are given in the Results.

The K ion activity of each internal solution was measured with a K-sensitive electrode (model 93-19; Orion Research Inc., Cambridge, MA). Negligible variation in the measured K activity (~ 270 mM) was observed among the various salts tested. The values determined directly agreed well with several values calculated in Robinson and Stokes (1965).

Junction potentials between SIS and the various internal solutions are listed in Table I and were compensated in plots of current-voltage relations. The temperature in all experiments was maintained between 10 and 12°C ($\pm 0.1^\circ\text{C}$) with a Peltier device and electronic feedback circuitry.

RESULTS

Exchanging the internal solution of a perfused squid axon from 320 KGlu to 320 KF results in a slow, progressive decline in the magnitude of potassium currents seen with depolarizing voltage steps. The rate of decline is variable during an observation period of 30–60 min, and appears to depend upon the rate of internal perfusion. Potassium current families for an axon before (A) and after (B) 30 min of perfusion with 320 KF are illustrated in Fig. 1. In addition, families of currents are shown for another axon during perfusion with aspartate (C) and chloride (D) anions. The currents are depressed by $\sim 70\%$ in B and the rate of activation of g_K is greatly decreased, as evidenced by the very slow rise of I_K during each depolarization in 320 KF. During longer depolarizations, the slowly rising K currents eventually reach a steady level. This change in the nature of the delayed potassium currents is not simply due to deterioration, as no such changes were observed during perfusion with 320 KGlu for >1 h.

Fluoride Effects Are Reversible and Specific for K Channels

These effects of fluoride perfusion are both specific for potassium channels and reversible, as shown in Fig. 2. After a 50-min exposure to 320 KF, the K currents are markedly suppressed (B), whereas the Na currents are unaltered. Upon a brief return to a glutamate-containing solution (320 KGlu), the K currents are substantially recovered (C).

The slow time course of the fluoride suppression of K current is illustrated in Fig. 3A for an axon in which Na current was not blocked by TTX. The decline in I_K is seen to progress throughout the 50-min exposure period. The rate of decline of K current amplitude and slowing of kinetics was independent of test membrane potential and of the frequency of channel activation by repetitive pulses. Unfortunately, no experiments were continued sufficiently long to determine whether I_K could be completely eliminated in 320 KF. The slow time course of block does not reflect internal solution exchange times, as seen by comparison with the recovery time. In contrast to the rate

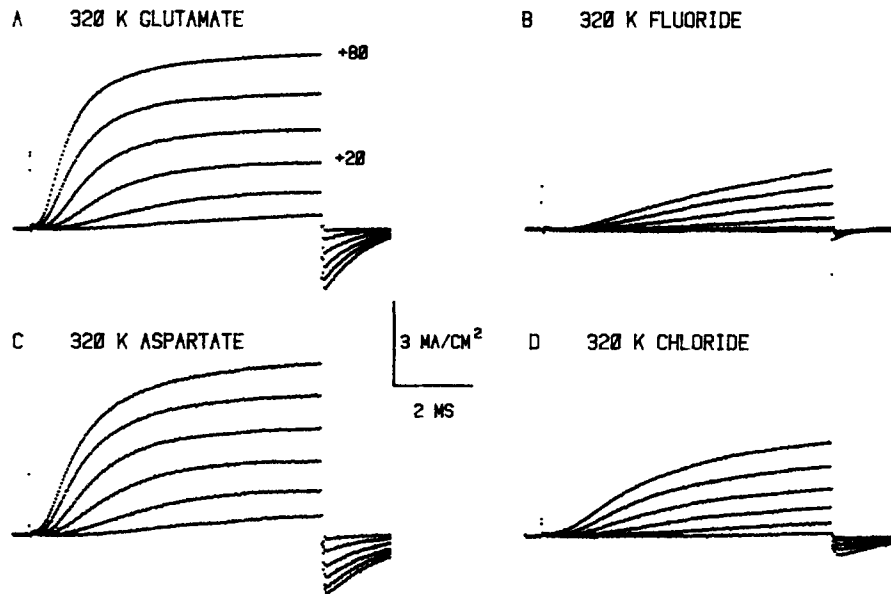


FIGURE 1. Suppression of delayed rectifier K currents in a squid axon perfused with high internal fluoride. (A) Potassium current records for 8-ms depolarizations from -20 to $+80$ mV in a squid axon perfused with 320 K Glu and bathed in ASW containing 300 nM TTX. (B) K currents from the same axon after 30 min of perfusion with 320 KF, at 10°C . (C) K currents from another axon perfused with 320 mM K aspartate for voltage steps from -20 to $+80$ mV. (D) K currents from the same axon as in C after 25 min of perfusion with 320 mM KCl at 10.2°C .

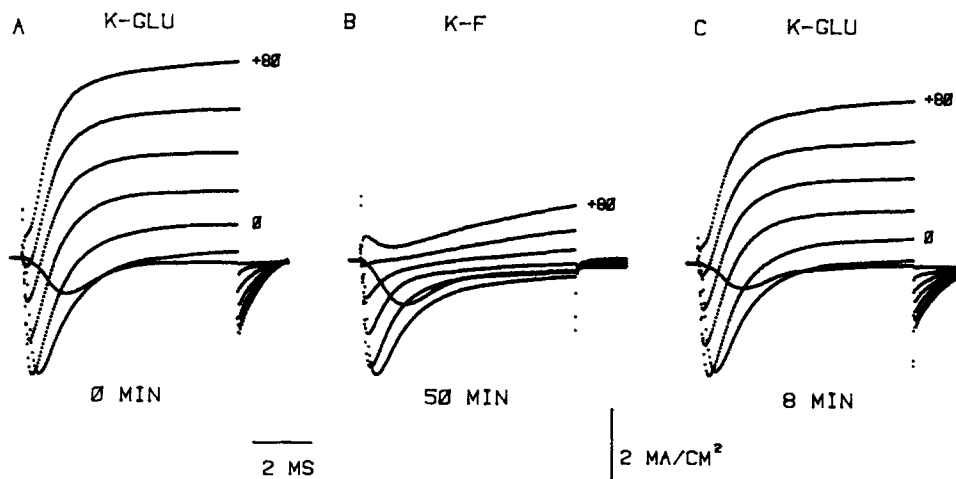


FIGURE 2. Anion suppression of I_K is specific and reversible. (A) Na and K currents recorded from a squid axon bathed in ASW and perfused with 320 K Glu. (B) Ionic currents from the same axon after a 50-min perfusion with 320 KF. (C) Currents measured again 8 min after return to 320 K Glu perfusion. Note stability of I_{Na} , but lability of I_K .

of I_K decline, the recovery upon returning to 320 KGlu is very rapid, reaching a steady state within 1 min after the solution change (Fig. 3B). Most of the delay seen during recovery, however, probably does reflect solution exchange time. Again, it can be seen that the Na current is unchanged during these procedures.

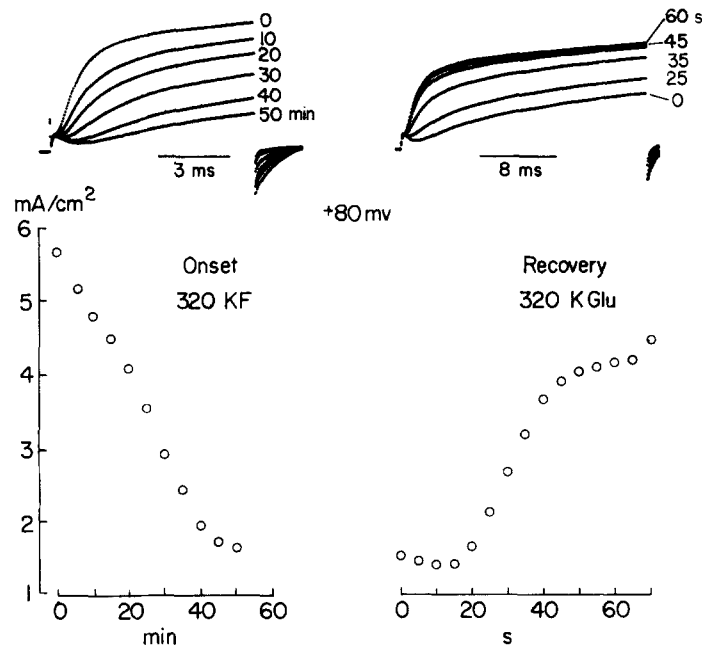


FIGURE 3. Time course of onset of and recovery from the internal fluoride effect on I_K . (A) Upper records are superposed measurements of I_{Na} and I_K at +80 mV obtained at the indicated times after beginning perfusion with 320 KF. The lower graph represents the time course of reduction of I_K measured at the end of each voltage step. (B) Records of the recovery of I_K at different intervals after 320 KGlu perfusion. Note the different times scales for the graphs in A and B.

The steady state recovery of K current was also examined in experiments in which the ratio of internal fluoride to glutamate was varied. After a substantial reduction of I_K had been achieved by perfusion with 320 KF, a significant recovery was induced by perfusion with a solution containing 318.4 mM KF and only 1.6 mM K glutamate (F/Glu ratio = 200:1) (Fig. 4A). The steady state levels of I_K recovery in other F/Glu ratios are also shown in Fig. 4A for this axon.

Ratios of F/Glu >1.0 are not required to produce the suppression of K current. Large increases ($\geq 30\%$) in I_K were always observed upon replacement of SIS (F/Glu = 0.16) by 320 KGlu (see Table II). Fig. 4B illustrates this characteristic increase in I_K in an axon perfused with SIS followed by 320 KGlu and then the decrease in I_K after perfusion with 320 KF. It is possible,

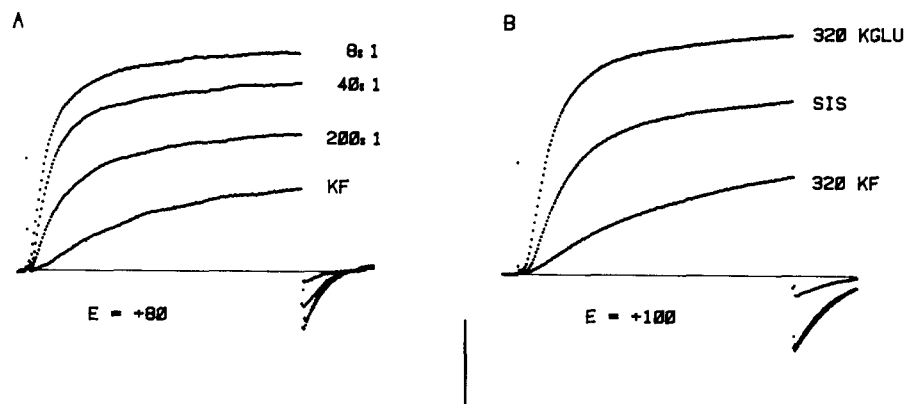


FIGURE 4. Recovery of fluoride-suppressed I_K by the addition of various internal glutamate concentrations. (A) Restoration of I_K by solutions of the indicated ratios of fluoride/glutamate after exposure to 320 KF (trace marked KF). (B) K current records (+100 mV) for an axon sequentially perfused with SIS, 320 KGLu, and 320 KF. Note the increase in I_K during 320 KGLu perfusion. 300 nM TTX was used in ASW to block I_{Na} .

though unlikely, that the increased current seen after SIS replacement represents a removal of block by the 50 mM Na^+ in SIS because control experiments adding this amount of Na to fluoride-free solutions reduced currents by only 5%.

Fluoride Prolongs the Action Potential

Under current-clamp conditions the duration of the membrane action potential is progressively increased ($\sim 30\%$) upon exposure to internal 320 KF. Upon return to 320 KGLu the duration returns to the control value. Upon replacement of SIS with 320 KGLu the action potential becomes shorter in

TABLE II
Effect of Internal Anions on the K Channel

	Anion	g_K	dg_K/dt	g_L
Organics	Glutamate ⁻	+	+	0
	Aspartate ⁻	+	+	0
	Citrate ³⁻	++	+	+
	Isethionate ⁻	-	-	0
	MOPS ⁻	-	-	0
Inorganics	F ⁻	-	-	0
	Cl ⁻	-	-	+
	Br ⁻	-	-	++
	NO ₃ ⁻	-	-	+
	SO ₄ ²⁻	-	---	0
	PO ₄ ²⁻	-	-	0

+, increase; -, decrease; 0, unchanged.

Favorability of internal anions for the squid axon K channel with respect to the standard internal perfusion solution (SIS); 320 mM K glutamate, 50 mM NaF, 300 mM sucrose, 15 mM KH₂PO₄ buffer, pH 7.3.

duration. Such changes are expected from the observed suppression of I_K in the presence of internal fluoride. Similar changes in action potential duration can be seen in the records of Adelman et al. (1966, Fig. 3).

Inorganic Anions Reduce and Slow g_K

11 different anions were tested in internal perfusion solutions with regard to their suitability for sustaining a large, rapidly activating g_K . The relative favorability of the various anion solutions is indicated in Table II with respect to SIS. In general it was found that the organic anion tested could maintain g_K during prolonged perfusion (>60 min). In contrast, all inorganic anions examined reversibly reduced both the amplitude and activation rate (dI_K/dt) of potassium current. Some of the inorganic species (e.g., NO_3 and Br) appeared to produce the most dramatic effects. The effects of all anions were readily reversible upon return to the control solution. No significant changes in leakage conductance (g_L) were observed between solutions containing glutamate and fluoride, whereas reversible increases in g_L were seen during perfusion with NO_3 , Br, or citrate. Since the degree and rate of these effects vary among axons with different rates of perfusion, a rigorous, quantitative comparison and ranking of the anions is not yet possible.

Inorganic Anions Slow g_K Activation But Not Deactivation

Upon substitution of fluoride for glutamate in the internal solution, the turn-on kinetics of I_K are dramatically slowed. The degree of slowing varies among experiments in axons with differing rates of perfusion and is therefore somewhat difficult to quantitate but is substantial and clear in all cases. From several measurements of the maximum rate of rise of I_K during a voltage step (dI_K/dt), fluoride slowed I_K turn-on by between 3- and 10-fold. In Fig. 5 the time course of K current at +80 mV is illustrated for an axon perfused with 320 KGlu (A) and then with 320 KF (B). Note that the vertical scale in B has been increased two times. The slowing of the rate of g_K activation by fluoride is evidenced both by comparing the rates of I_K turn-on during the test pulse and by comparing the envelopes of tail currents upon repolarization to -80 mV from different test pulse durations.

Potassium currents were recorded in either 320 KF or 320 KGlu at several different test potentials after conditioning prepulses to between -80 and -160 mV. A comparison of the records in the two solutions was made by vertically scaling the smaller, slower record in fluoride at a given potential to visually parallel the time course of the larger record obtained in glutamate at the region of maximum dI_K/dt . The two records compared in this manner nearly superimposed during this rapid activation phase, which suggests that the change in the time course of g_K activation by fluoride reflects primarily a slowing of channel opening rates and not an additional delay of the initial g_K turn-on.

In contrast to the marked slowing of activation, the turn-off of g_K upon repolarization (deactivation) is not affected by changes in the internal anion composition. In Fig. 5C tail currents from an axon internally perfused with

four different anions are arbitrarily scaled to match the amplitude of the tail current in the presence of glutamate. The time constants for single exponentials fit to each record are given and demonstrate the relative constancy of the rate of deactivation in the four conditions. In fact, the tail current in fluoride is slightly faster than in glutamate, in contrast to the slowing observed in the activation rate. Potassium tail current time constants obtained at several values of membrane potential are plotted in Fig. 6 for the four anion species.

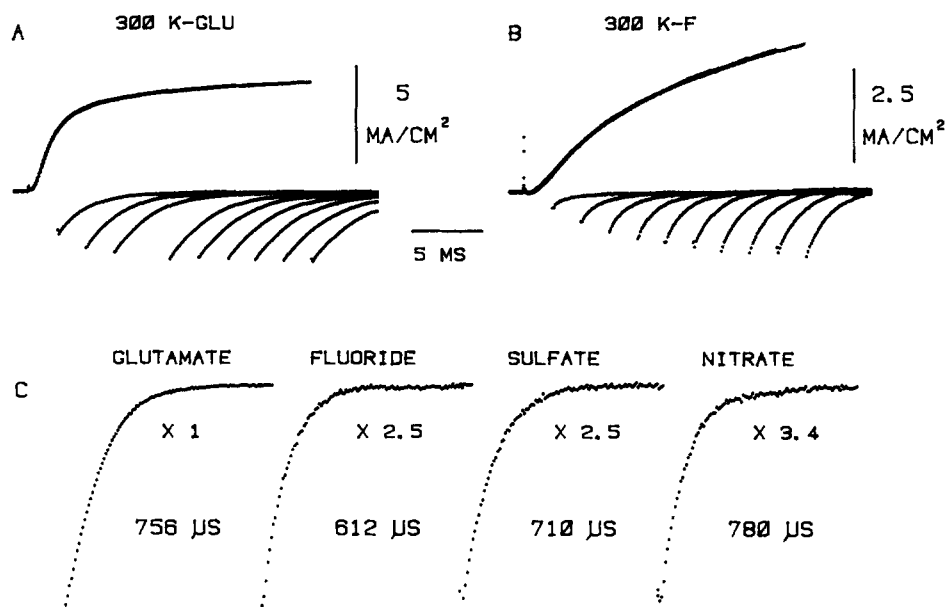


FIGURE 5. Effect of anion substitution on K channel kinetics. (A) I_K at +80 mV in an axon perfused with 320 K-Glu for steps of increasing duration. The activation time course can be observed from the rise of current during the step and from the envelope of the tail currents upon return to the holding potential of -80 mV. (B) The same measurements performed on the axon after perfusion with 320 K-F. Note the increase in vertical gain. (C) K tail currents at -140 mV after a 6-ms prepulse to +60 mV in perfusates of different compositions as indicated. The currents have been scaled vertically to the same amplitude and the scale factors and time constants for single exponentials fit to the tails are given in each case.

In these experiments HKSU was used to minimize K ion accumulation/depletion problems that might contaminate the tail current records and result in multicomponent time courses. It can be seen that no significant changes in the time course of tail currents were seen over this potential range.

Instantaneous I-V Relations in Different Internal Anions

The instantaneous conductance of open potassium channels was determined in the presence of different internal anions from instantaneous current-voltage data. Brief (4–6 ms) conditioning voltage steps were applied to the

axon membrane and the potential was then stepped to voltages between -160 and $+140$ mV. "Instantaneous" K currents were measured $200 \mu\text{s}$ after the end of the conditioning step (leakage and capacitive currents were electronically subtracted). Representative I_K - V curves are shown in Fig. 7 for four different internal anion solutions. The K conductance in the presence of each of the inorganic anions (F^- , SO_4^{2-} , NO_3^-) is smaller than that observed in the organic anion (glutamate). The reversal potential (corrected for junction potentials) remains constant throughout the changes in internal solution, which suggests that none of the anions were measurably permeant through K channels.

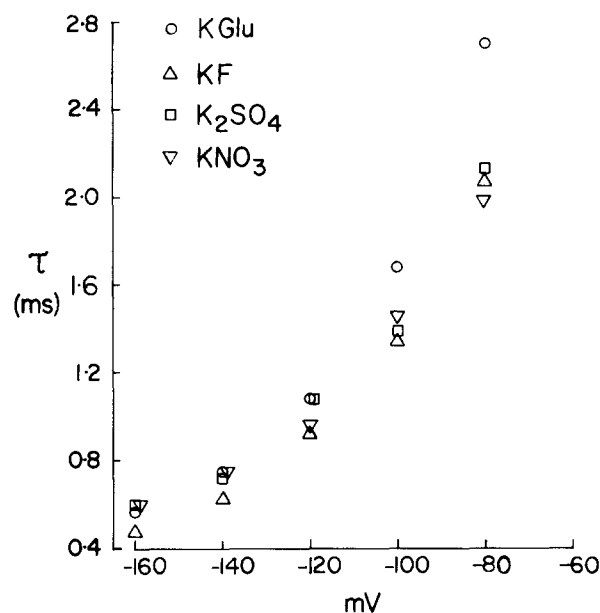


FIGURE 6. Time constants for K tail currents as a function of membrane potential in the presence of four different internal anions.

The reduction in open-channel conductance during perfusion with the "less favorable" anions is not voltage-dependent. The smooth line through the data points in 320 K Glu (Fig. 7) is fit by eye, whereas that through the remaining data is the same line scaled by a factor of 0.43. This suggests that the anion-K channel interaction does not involve a blocking action at a binding site located in the ion pathway an appreciable distance into the membrane electric field, as proposed for other molecules (e.g., French and Shoukimas, 1981).

The effect of internal fluoride on g_K is independent of the concentration of external potassium. Fluoride effects were maintained in four different external K concentrations (0, 10, 220, and 445 mM) and were not qualitatively different, which further suggests that F^- ions are not directly in a

pathway subject to “knock-on” interactions with K ions (Armstrong, 1975). Small quantitative differences in the onset rate of the effect on different potassium concentrations are hard to evaluate because of the degree of variability in the time course and need further examination.

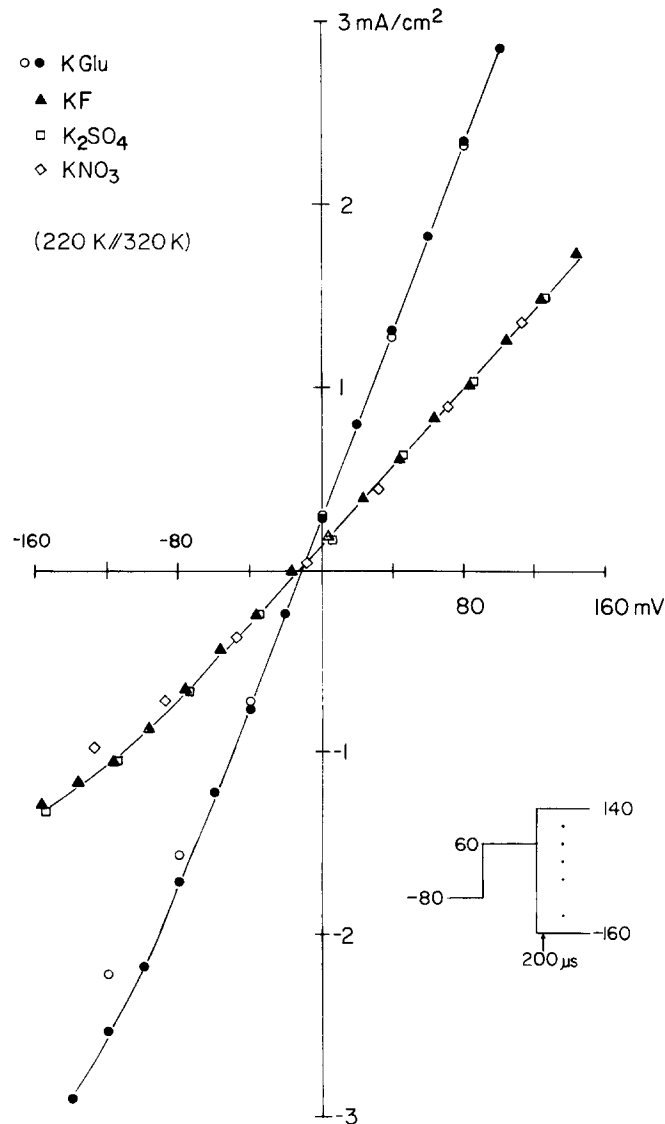


FIGURE 7. Instantaneous current-voltage relationships of K channels in four different internal anions. Currents were measured at the indicated potentials 200 μ s after a prepulse to +60 mV. The external solution contained 220 mM K⁺ to minimize accumulation effects. The data are corrected for junction potentials.

Cationic Blockers of K Channels Antagonize Anion Effects

Despite the evidence just mentioned, which suggests that the site of anion action is not in the interior ion pathway of the K channel, certain experiments argue that the site is, nonetheless, close to the inner "mouth" of the channel. We examined the interaction between the anion effect and certain classic cationic blockers of voltage-dependent K channels: tetraethylammonium ion (TEA^+), Cs^+ , and 4-aminopyridine (4-AP^+). Fig. 8 illustrates the effects of addition of $100\ \mu\text{M TEA}^+$ to the internal solutions of axons perfused with either $320\ \text{KGLu}$ (A) or $320\ \text{KF}$ (B). TEA^+ reduced I_K in KGLu by 23% with no change in channel kinetics (A). This is in agreement with previous

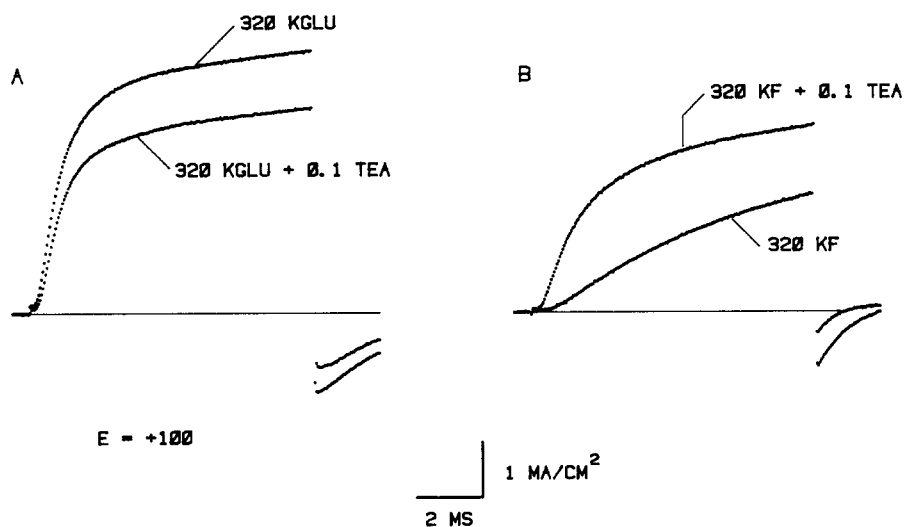


FIGURE 8. Paradoxical relief of anion effect by TEA^+ . (A) K currents recorded at $+100\ \text{mV}$ in a $320\ \text{KGLu}$ -perfused axon before (upper) and after (lower) the addition of $100\ \mu\text{M TEA}^+\text{Br}^-$ to the solution. (B) K currents recorded from the same axon after perfusion with $320\ \text{KF}$ alone (lower) and after the addition of $100\ \mu\text{M TEA}^+\text{Br}^-$ to the solution (upper). Note the increase in I_K upon addition of TEA^+ .

observations (see Armstrong, 1975) and with an apparent dissociation constant of $300\ \mu\text{M}$ for TEA^+ block of K channels in squid axons determined in separate experiments (G. S. Oxford, unpublished observations).

In contrast, addition of $100\ \mu\text{M TEA}^+$ (in KF) to the same axon during $320\ \text{KF}$ perfusion (B) increased both the current amplitude and the initial dI_K/dt . Although higher concentrations of TEA^+ ($1\ \text{mM}$) caused partial reduction of I_K in KF, an increase in the rate of activation was still observed. Similar antagonism of the fluoride effect was seen with the addition of $40\ \mu\text{M}$ 4-AP (Yeh et al., 1976a, b) or $10\text{--}50\ \text{mM Cs}^+$ (Bezanilla and Armstrong, 1972) to the internal 320-KF solution.

It is reasonable to imagine that the recovery of rapid K channel activation with cationic blockers is related only to their charge and relative size rather

than their specific interactions with the channel. We sought to examine this possibility by adding an "inert" cation to the perfusion solution. A cation would be considered inert with respect to the K channel if it was not measurably permeant through the open channel or if it did not block K^+ movement through the channel. In separate experiments, *N*-methylglucamine (NMG) was found to approximately satisfy these criteria (Oxford and Adams, 1981), as it cannot pass through the channel and demonstrates slight blocking action only at relatively high concentrations (>100 mM internally). Addition of 0.1–10 mM NMG^+F^- after an extended perfusion with KF failed to induce any reversal of the anion effect. Thus, to our knowledge, only cations with blocking actions on K channels are capable of triggering a recovery from the depression and slowing of g_K induced by fluoride perfusion. Further validation of this proposal must await the development of a truly inert cation substitute.

K Channels Can Survive in the Absence of K^+

The effect of internal perfusion with F^- ions on I_K is reminiscent of the after-effects of NaF perfusion on K currents described by Chandler and Meves (1970). Similar observations have been extended by Almers and Armstrong (1980) to suggest that potassium channels require the presence of a permeant cation on at least one side of the membrane to retain their normal conducting properties. To determine whether, in fact, the loss of K current observed under these conditions reflects the presence of Na, the absence of K, or the nature of the anionic species used, we repeated the experiment of Chandler and Meves (1970, Figs. 11 and 12) using internal Na glutamate rather than NaF. In Fig. 9 ionic currents at several membrane potentials are shown from an axon in 320 KGlu (A), after 50 min of perfusion with 320 NaGlu (B), and subsequently upon return to 320 KGlu (C). The kinetics of K current were unchanged after Na perfusion, as shown by the comparison of scaled records at +80 mV in Fig. 9D. The 35% reduction in I_K observed here can be compared with an $\sim 90\%$ reduction observed by Chandler and Meves (1970). In a separate experiment with 320 NaGlu perfusion of an axon in K-free seawater, I_K was seen to fall by only 10% after 30 min. This can be compared with reductions of $>90\%$ seen during comparable exposures of axons to K-free media in the presence of 50 mM internal fluoride (Almers and Armstrong, 1980). Thus, it appears that the presence of internal fluoride ions may play a major role in the phenomenon of g_K loss during perfusion with solutions free of K channel permeant cations. An alternative possibility is that the small amount of phosphate buffer that we used throughout our experiments is involved in the K channel protective effect, as phosphate was not used in either of the previous studies.

Anion Effects Are Not Due to Heavy-Metal Contamination or Internal Potential Changes

We have suggested that fluoride and other inorganic anions interact with a site on the intracellular surface of K channels to suppress channel opening.

An alternative explanation might involve the presence of heavy-metal contaminants (primarily Pb^{++}) found in these potassium salts. Total Pb^{++} contamination was estimated to be $\sim 2-6 \mu M$ in a solution of 320 KF. To examine this possibility, axons were perfused with 320 KF with the addition of 0.1–1 mM EDTA. EDTA at these concentrations would be expected to effectively chelate any heavy metals present (stability constant of EDTA and $Pb^{++} = 10^{18}/\text{mol}\cdot\text{liter}$ [Sillen and Martell, 1971]). Under these conditions continuous perfusion for 30–60 min still resulted in a characteristic suppression and slowing of g_K . In a separate experiment, washing with EDTA-containing KF

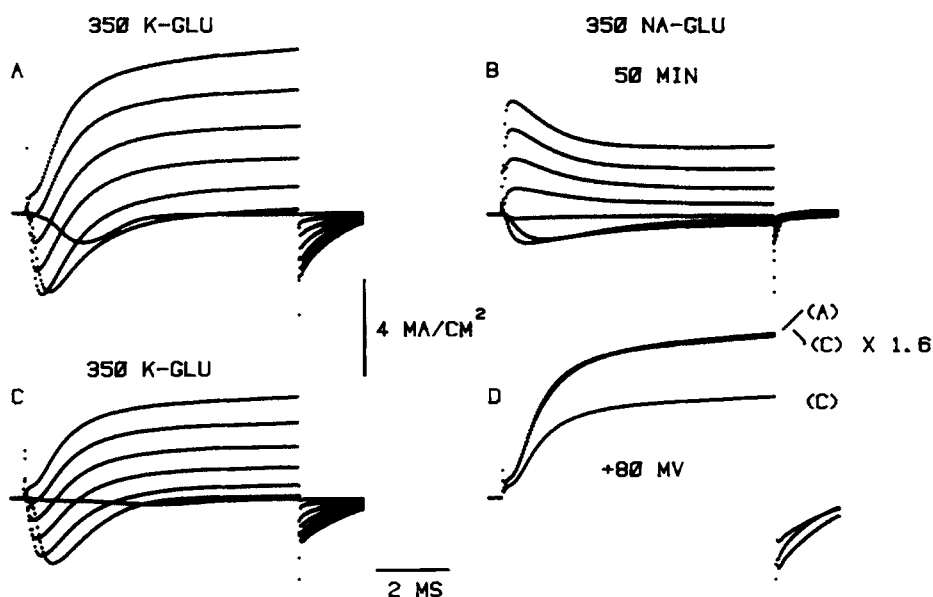


FIGURE 9. Significant survival of K channels after perfusion with Na glutamate solutions. (A) Family of ionic currents (Na and K) for an axon perfused with 320 KGlu and bathed in ASW. (B) Ionic current family from the same axon after 50 min of perfusion with 320 NaGlu. (C) Recovery of currents upon return to 320 KGlu. (D) Superposed records of currents at +80 mV from the families in A and C plus a scaled ($\times 1.6$) record from C.

perfusate after exposure to 320 KF alone also failed to restore the suppressed g_K .

Another possible explanation for the effects seen with internal perfusion with fluoride would invoke chelation of intracellular calcium ions remaining near the membrane surface by fluoride ions. Were this to occur, the K channels would experience an effective hyperpolarization because of the unmasking of fixed negative surface charges and subsequent alteration of internal surface potential. This possibility was ruled out by comparisons of the isochronal I_K -V relations in 320 KGlu and 320 KF, which revealed no

shifts along the voltage axis. The stability of the deactivation time constant vs. voltage curve (Fig. 6) further supports this point.

DISCUSSION

Our observations strongly suggest that the maintenance and survival of voltage-dependent potassium channels in the squid axon membrane are critically dependent upon the nature of the intracellular anion environment. Previous studies involving systematic changes in intracellular anions in squid axons have been confined to determining the survival of action potential generation as an index of "favorability" of internal anions (Baker et al., 1962; Tasaki et al., 1965). The latter authors determined that (excluding organic anions) the most favorable anions followed the lyotropic series with fluoride best supporting axon survival. As a result of this discovery and similar reports (Adelman et al., 1966), fluoride has served as the principal internal anion in many internal perfusion/dialysis studies of ionic channel function. Indeed, it is our experience and that of others (e.g., Adelman et al., 1966; Goldman and Kenyon, 1979) that a minimum level of intracellular fluoride is conducive to long-term maintenance of a functional sodium channel population during perfusion experiments. In contrast, it is obvious from the results presented here that even this level of fluoride (e.g., SIS) is not optimal for maintaining K channels.

A review of the literature involving the use of internal perfusion or exchange procedures to study ionic channels suggests that the presence and maintenance of outward K currents depends upon the choice of intracellular anion in a manner consistent with our observations. Adelman et al. (1966) found that transient sodium currents in squid axons perfused with 400 mM KCl solutions progressively decline and were restored by perfusion with 400 mM KF or maintained by 90% KCl plus 10% KF solutions. Although these authors report no observations on K currents, it appears from their records (Figs. 1 and 3) that I_K declined progressively during exposure to either KCl or KF. A broadening of the action potential can also be seen in their records.

During experiments on voltage-clamped single frog skeletal muscle fibers using the vaseline-gap technique, it was observed that normal outward K currents were diminished or totally absent when the ends of muscle fibers were cut in KF solutions (Hille and Campbell, 1976). In recent experiments using the same technique, Vergara et al. (1978) observed normal outward K currents in voltage-clamped muscle fibers when the ends were cut in a solution containing 120 mM aspartate⁻, 2–5 mM Cl⁻, and no fluoride. These results suggest that internal fluoride suppresses I_K in frog muscle as in squid axon.

Survival of K channel permeability has been linked to the presence of permeant cations on at least one side of the axon membrane (Almers and Armstrong, 1980). These studies followed an earlier report by Chandler and Meves (1970) that K currents were irreversibly depressed in K-free seawater after prolonged (>20 min) perfusion with NaF. In both studies the internal perfusion media contained fluoride anions. Our observation of substantial recovery of I_K after perfusion with glutamate and phosphate as the only

internal anions in the absence of K channel permeant cations suggests that a major fraction of the K current decline observed in previous studies may reflect internal anion effects. Recently, Schauf (1982) has also reported maintenance of K channels during dialysis of *Myxicola* axons with solutions containing no permeant cations as well as enhanced decline of K currents in fluoride-perfused axons.

The 11 different anions examined here provide a spectrum of chemical and geometrical properties as concerns protein interactions and ionic radii. Unfortunately, the slightly variable nature and slow time course of the effects seen from axon to axon preclude a quantitative comparison among them at this time. As a broad generalization, the organic anions appeared to maintain K currents, whereas all inorganic anions tested suppressed them to some degree. Further experiments are required to establish reliable criteria for direct comparisons among a series of anions.

Summary of Differences Between Fluoride- and Glutamate-perfused Axons

Our experiments have concentrated on a comparison of fluoride and glutamate as intracellular anions. The major experimental observation is a reversible suppression of outward I_K and a marked slowing of the activation of K channels in fluoride-perfused axons. These effects develop with a slow time course and are specific for K channels over Na channels. The slow rate of onset cannot be attributed to diffusional barriers restricting access because of the rapid internal solution exchange (80–100 $\mu\text{l}/\text{min}$) and because reversal of the effect occurs within seconds after replacing the internal glutamate solution. Prior perfusion with glutamate does not protect against the fluoride effect. The effect is independent of the external K^+ concentration and test membrane potential and does not recover with frequent application of depolarizing voltage steps, as observed with aminopyridine block of K channels (Yeh et al., 1976a, b). It appears that F^- does not act as do more typical K channel blocking agents such as quaternary ammonium compounds, Cs^+ , aminopyridines, or internal Ba^{++} (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980), which can exhibit a variety of voltage- and frequency-dependent interactions, but do not significantly alter intrinsic channel gating kinetics.

Fluoride ions exert a differential effect upon K channel gating kinetics. Activation of I_K during depolarizing steps is slowed dramatically, whereas deactivation kinetics following the step are either speeded slightly or not altered. Fluoride ions binding to sites at the internal membrane surface might be expected to hyperpolarize the membrane electric field via altered surface potentials and produce an apparent shift of the voltage-dependent rate constants for activation and deactivation to more depolarized potentials along the voltage axis. If these rate constants are of the traditional Hodgkin-Huxley (1952) form, a surface potential-induced shift would be qualitatively consistent with our observations; however, the much greater sensitivity of activation to fluoride exposure argues strongly against this simple mechanism. Additionally, no significant shift of the steady state I_K - V relation was observed in KF, which is also inconsistent with surface potential mechanisms.

Possible Mechanisms and Models of Anion Effects

The selectivity of fluoride effects for K channels and the slowing of channel opening suggest that F ions bind to a component of the K channel molecule that controls a conformational transition in the activation sequence. The contrast between the slow onset of and rapid recovery from the effect may reflect either a very small forward rate constant for binding or a slow transition of the channel protein to a new conformation after F⁻ binding. The stoichiometry of the effect is not clear and thus it is conceivable that a number of F ions bind to each channel, producing an incremental slowing for each bound ligand. Conceivably, if such sites were saturated, little or no activation of a K channel would take place. A determination of the concentration dependence of these effects is not straightforward because of the necessity of replacing anions rather than adding them to the perfusate.

The action of external Zn⁺⁺ on K channels similarly involves a dramatic slowing of activation (Stanfield, 1975) and it was recently observed in squid axons (Gilly and Armstrong, 1982) that although K channel activation was slowed by Zn⁺⁺, deactivation kinetics were negligibly altered. This differential action of Zn⁺⁺ was advanced as evidence for a specific binding of the divalent ion at or near a negatively charged gating component of the K channel that resides near the external membrane surface in the resting state (Gilly and Armstrong, 1982). A bound divalent cation would increase the activation energy for the opening gating transition by stabilizing the gating charge in the resting state. When the activation process does occur, the gating charge dissociates from the Zn ion to take a position toward the inner membrane surface in the open channel state free of bound Zn⁺⁺. Thus, its return to the resting position during deactivation would not be subject to the action of Zn⁺⁺ present only at the external surface and would presumably occur at a normal rate.

A variation on this scheme might be postulated for internal F ions where repulsive coulombic forces between bound F⁻ and negative gating charges could retard channel opening. Upon gate opening, F ions would be displaced from their binding site by the inward migration of negative gating charge(s) and thus would not be in a position to influence closing rates significantly.

Since the anion effects are confined to certain species, negative charge is not the only feature required for the effect. The ionic radius is perhaps a more important feature because most of the inorganic anions are much smaller than the organic anions examined. An ion of smaller effective radius could conceivably have better access to a "partially buried" site near the inner channel surface. Further studies will have to be conducted to clarify those properties of the anions important for these effects.

Our results do, however, provide evidence that the action of F⁻ can be influenced by the binding of several well-characterized cationic molecules that block K channels to their binding site(s), which is thought to reside nearest the inner "mouth" of the channel. The recovery of I_K amplitude and kinetics upon introducing low concentrations of TEA⁺, Cs⁺, or 4-AP⁺ (but not NMG⁺) implies that the binding site for F⁻ is perhaps a component of

the receptor(s) for these agents and may even reside partially within the K channel ion pathway itself.

Consequences of the Present Findings

The observation that some anions profoundly alter K channel gating when present internally and the antagonism of the effect by internal cationic blockers suggest that at least part of the K channel gating mechanism resides near the inner surface of the channel. This conclusion is consistent with previous evidence for an inner gate site based upon studies of TEA⁺ block in K channels (e.g., Armstrong and Hille, 1972).

One interpretation of the paradoxical antagonism of F⁻ effects by cationic channel blockers, but not by inert cations, is that an anion may indeed partially enter an "exclusively" cation-selective channel. Such a suggestion is not, however, new to the study of membrane channels. Studies of the relationship between cation conductance and activity in gramicidin channels have suggested a significant occupancy of these channels by anions coincident with high occupancy by thallos ions (Sandblom et al., 1977; Eisenman et al., 1978). In this case a cooperative binding of anions to gramicidin channels appears to facilitate multioccupancy by other cations. As it is possible that the F⁻-TEA⁺ interaction reflects an allosteric alteration of the channels, a new site for pharmacological modification of K channels may be represented by anion effects.

It is clear that attention must be given to the anion composition as well as the cation composition of artificial intracellular media during cell perfusion or isolated membrane patch experiments. The determination of average single K channel closed times during exposure to F⁻-containing solutions can be expected to yield artificially large values.

The authors wish to express their appreciation to Drs. J. Z. Yeh, C. H. Wu, and M. Johnston for performing "blind" experiments in verification of our initial results. Thanks are again due to Dr. Wu for assistance in performing the EDTA experiments and to Dr. Bruce Kagan for measurements of K ion activities in our solutions.

This work was supported by a National Science Foundation grant (BNS79-21505) to G.S.O. and a Grass Foundation Fellowship to D.J.A.

Received for publication 26 August 1982 and in revised form 3 June 1983.

REFERENCES

- Adams, D. J., and G. S. Oxford. 1981. Internal anions modulate potassium channel gating in squid axons. *Biophys. J.* 33:70a. (Abstr.)
- Adelman, W. J., F. M. Dyro, and J. P. Senft. 1966. Internally perfused axons: effects of two different anions on ionic conductance. *Science (Wash. DC)*. 151:1392-1394.
- Almers, W., and C. M. Armstrong. 1980. Survival of K⁺ permeability and gating currents in squid axons perfused with K⁺-free media. *J. Gen. Physiol.* 75:61-78.
- Armstrong, C. M. 1975. Potassium pores of nerve and muscle membranes. In *Membranes. Dynamic Properties of Lipid Bilayers and Biological Membranes: A Series of Advances*. G. Eisenman, editor. Marcel Dekker, Inc., New York. 3:325-358.

- Armstrong, C. M., and B. Hille. 1972. The inner quaternary ammonium ion receptor in potassium channels of the node of Ranvier. *J. Gen. Physiol.* 59:388-400.
- Armstrong, C. M., and S. R. Taylor. 1980. Interaction of barium ions with potassium channels in squid giant axons. *Biophys. J.* 30:473-488.
- Baker, P. F., A. L. Hodgkin, and T. I. Shaw. 1962. Replacement of the axoplasm of giant nerve fibres with artificial solutions. *J. Physiol. (Lond.)*. 164:330-354.
- Begenisich, T., and M. D. Cahalan. 1980. Sodium channel permeation in squid axons. I. Reversal potential experiments. *J. Physiol. (Lond.)*. 307:217-242.
- Bergman, C. 1970. Increase of sodium concentration near the inner surface of the nodal membrane. *Pflügers Arch. Eur. J. Physiol.* 317:287-294.
- Bezanilla, F., and C. M. Armstrong. 1972. Negative conductance caused by entry of sodium and cesium ions into the potassium channels of squid axons. *J. Gen. Physiol.* 60:588-608.
- Binstock, L., W. J. Adelman, J. P. Sényi, and H. Lecar. 1975. Determination of the resistance in series with the membranes of giant axons. *J. Membr. Biol.* 21:25-47.
- Cahalan, M. D., and T. Begenisich. 1976. Sodium channel selectivity: dependence on internal permeant ion concentration. *J. Gen. Physiol.* 68:111-125.
- Chandler, W. K., and H. Meves. 1970. Sodium and potassium currents in squid axons perfused with fluoride solutions. *J. Physiol. (Lond.)*. 211:623-652.
- Conti, F., and E. Neher. 1980. Single channel recordings of K⁺ currents in squid axons. *Nature (Lond.)*. 285:140-143.
- Deffner, G. G. J. 1961. The dialyzable free organic constituents of squid blood; a comparison with squid axoplasm. *Biochim. Biophys. Acta.* 47:378-388.
- Eaton, D. C., and M. S. Brodwick. 1980. Effects of barium on the potassium conductance of squid axon. *J. Gen. Physiol.* 75:727-750.
- Ebert, G. A., and L. Goldman. 1976. The permeability of the sodium channel in *Myxicola* to the alkali cations. *J. Gen. Physiol.* 68:327-340.
- Eisenman, G., J. P. Sandblom, and E. Neher. 1978. Interactions in cation permeation through the gramicidin channel: Cs, Rb, K, Na, Li, Tl, H, and the effects of anion binding. *Biophys. J.* 22:307-340.
- French, R. J., and J. J. Shoukimas. 1981. Blockage of squid axon potassium conductance by internal tetra-*n*-alkylammonium ions of various sizes. *Biophys. J.* 34:271-292.
- French, R. J., and J. B. Wells. 1977. Sodium ions as blocking agents and charge carriers in the potassium channel of the squid giant axon. *J. Gen. Physiol.* 70:707-724.
- Gilly, W. F., and C. M. Armstrong. 1982. Divalent cations and the activation kinetics of potassium channels in squid giant axons. *J. Gen. Physiol.* 79:965-996.
- Goldman, L., and J. L. Kenyon. 1979. Internally perfused *Myxicola* giant axons showing long-term survival. *Biophys. J.* 28:357-362.
- Hille, B., and D. T. Campbell. 1976. An improved vaseline gap voltage clamp for skeletal muscle fibers. *J. Gen. Physiol.* 67:265-293.
- Hodgkin, A. L., and A. F. Huxley. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. (Lond.)*. 117:500-544.
- Horn, R., J. Patlak, and C. F. Stevens. 1981. The effect of tetramethylammonium on single sodium channel currents. *Biophys. J.* 36:321-327.
- Oxford, G. S. 1981. Some kinetic and steady-state properties of sodium channels after removal of inactivation. *J. Gen. Physiol.* 77:1-22.
- Oxford, G. S., and D. J. Adams. 1981. Permeant cations alter K channel kinetics and permeability. *Biophys. J.* 33:70a. (Abstr.)

- Oxford, G. S., C. H. Wu, and T. Narahashi. 1978. Removal of sodium channel inactivation in squid giant axons by *N*-bromoacetamide. *J. Gen. Physiol.* 71:227-247.
- Oxford, G. S., and J. Z. Yeh. 1979. Interference with sodium inactivation gating in squid axons by internal monovalent cations. *Biophys. J.* 25:195a. (Abstr.)
- Robinson, R. A., and R. H. Stokes. 1965. *Electrolyte Solutions*. Butterworth and Co. Ltd., London. 571 pp.
- Sandblom, J., G. Eisenman, and E. Neher. 1977. Ionic selectivity, saturation, and block in gramicidin A channels. I. Theory for the electrical properties of ion selective channels having two pairs of binding sites and multiple conductance states. *J. Membr. Biol.* 31:383-417.
- Schauf, C. L. 1982. Survival of the K⁺ channel in axons externally and internally perfused with K⁺-free media. *Biophys. J.* 40:171-173.
- Schauf, C. L., and J. O. Bullock. 1978. Internal cesium alters sodium inactivation in *Myxicola*. *Biophys. J.* 23:473-477.
- Sillen, L. G., and A. E. Martell. 1971. *Stability Constants of Metal-Ion Complexes*. Special publication No. 25. The Chemical Society, London.
- Stanfield, P. R. 1975. The effect of zinc ions on the gating of the delayed potassium conductance of frog sartorius muscle. *J. Physiol. (Lond.)* 251:711-735.
- Tasaki, I., I. Singer, and T. Takenaka. 1965. Effects of internal and external ionic environment on excitability of squid giant axon: a macromolecular approach. *J. Gen. Physiol.* 48:1095-1123.
- Vergara, J., F. Bezanilla, and B. M. Salzberg. 1978. Nile blue fluorescence signals from cut single muscle fibers under voltage or current clamp conditions. *J. Gen. Physiol.* 72:775-800.
- Yeh, J. Z., G. S. Oxford, C. H. Wu, and T. Narahashi. 1976a. Interactions of aminopyridines with potassium channels of squid axon membranes. *Biophys. J.* 16:77-81.
- Yeh, J. Z., G. S. Oxford, C. H. Wu, and T. Narahashi. 1976b. Dynamics of aminopyridine block of potassium channels in squid axon membrane. *J. Gen. Physiol.* 68:519-535.