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

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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...
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 Begenisich, 1976; Ebert and Goldman, 1976; Begenisich 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 (~80 μl/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. Begenisich (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 (HKSW) contained 220 mM

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Composition of Internal Solutions</th>
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<tbody>
<tr>
<td>Solution*</td>
<td>Anion</td>
</tr>
<tr>
<td>SIS</td>
<td>Glutamate</td>
</tr>
<tr>
<td>320 NaGlu</td>
<td>Glutamate</td>
</tr>
<tr>
<td>320 KGlue</td>
<td>Glutamate</td>
</tr>
<tr>
<td>320 KAsp</td>
<td>Aspartate</td>
</tr>
<tr>
<td>320 KF</td>
<td>Fluoride</td>
</tr>
<tr>
<td>320 KCl</td>
<td>Chloride</td>
</tr>
<tr>
<td>320 KBr</td>
<td>Bromide</td>
</tr>
<tr>
<td>320 KNO₃</td>
<td>Nitrate</td>
</tr>
<tr>
<td>160 K₂PO₄</td>
<td>Phosphate</td>
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<tr>
<td>160 K₂SO₄</td>
<td>Sulfate</td>
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<tr>
<td>320 KIs</td>
<td>Isethionate</td>
</tr>
<tr>
<td>107 K₃Cit</td>
<td>Citrate</td>
</tr>
<tr>
<td>506 KMOPO₅</td>
<td>MOPS</td>
</tr>
</tbody>
</table>

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

TL Table 1 continued...

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 (HPO₄²⁻) 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 HPO₄ concentration occurring naturally in Loligo pealei axoplasm (Deffner, 1961). The osmolarity of the solutions was adjusted to ~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 (±0.1°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 ~70% in B and the rate of activation of gK is greatly decreased, as evidenced by the very slow rise of IK 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 IK 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 IK 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 KGlut 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 KGlut. (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 KGlut perfusion. Note stability of $I_{Na}$, but lability of $I_k$. 
of $I_K$ decline, the recovery upon returning to 320 KGlue 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 KGlue 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 (≥30%) in $I_K$ were always observed upon replacement of SIS ($F$/Glu = 0.16) by 320 KGlue (see Table II). Fig. 4B illustrates this characteristic increase in $I_K$ in an axon perfused with SIS followed by 320 KGlue and then the decrease in $I_K$ after perfusion with 320 KF. It is possible,
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 (~30%) upon exposure to internal 320 KF. Upon return to 320 KGluthe duration returns to the control value. Upon replacement of SIS with 320 KGluthe action potential becomes shorter in

<table>
<thead>
<tr>
<th>Anion</th>
<th>$g_K$</th>
<th>$dg_K/dt$</th>
<th>$g_L$</th>
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<tbody>
<tr>
<td>Organics</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Glutamate⁻</td>
<td>+</td>
<td>+</td>
<td>0</td>
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<tr>
<td>Aspartate⁻</td>
<td>+</td>
<td>+</td>
<td>0</td>
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<tr>
<td>Citrate⁵⁻</td>
<td>++</td>
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<td>Isethionate⁻</td>
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<tr>
<td>MOPS⁻</td>
<td>−</td>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>Inorganics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F⁻</td>
<td>−</td>
<td>−</td>
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<td>Cl⁻</td>
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<td>SO₄²⁻</td>
<td>−</td>
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<td>0</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>−</td>
<td>−</td>
<td>0</td>
</tr>
</tbody>
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+, 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 7.5.
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.

In these experiments HKSW 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 μs after 
the end of the conditioning step (leakage and capacitative 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 junc-
tion potentials) remains constant throughout the changes in internal solution, 
which suggests that none of the anions were measurably permeant through 
K channels.

![Time constants for K tail currents as a function of membrane potential in the presence of four different internal anions.](image)

**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 KGl (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 

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 qualita-
tively 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 μs 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	extsuperscript{+}), Cs	extsuperscript{+}, and 4-aminopyridine (4-AP	extsuperscript{+}). Fig. 8 illustrates the effects of addition of 100 µM TEA	extsuperscript{+} to the internal solutions of axons perfused with either 320 KGlucose (A) or 320 KF (B). TEA	extsuperscript{+} reduced $I_K$ in KGlucose by 23% with no change in channel kinetics (A). This is in agreement with previous observations (see Armstrong, 1975) and with an apparent dissociation constant of 300 µM for TEA	extsuperscript{+} block of K channels in squid axons determined in separate experiments (G. S. Oxford, unpublished observations).

In contrast, addition of 100 µM TEA	extsuperscript{+} (in KF) to the same axon during 320 KF perfusion (B) increased both the current amplitude and the initial $dI_K/dt$. Although higher concentrations of TEA	extsuperscript{+} (1 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 µM 4-AP (Yeh et al., 1976a, b) or 10–50 mM Cs	extsuperscript{+} (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 gK 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 ~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 gK 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 ~2–6 μM 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++ = $10^{19}$/mol·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 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 KGl 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 μl/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 thallous 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.

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