MODULATION OF AMINOPYRIDINE BLOCK OF POTASSIUM CURRENTS IN SQUID AXON

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ABSTRACT Aminopyridines are known to block potassium (K) currents in excitable membranes in a manner dependent upon membrane potential, such that the block is relieved by depolarization and restored upon repolarization. In the present study, the effects of aminopyridines on voltage-dependent potassium (K) channels were examined in internally perfused, voltage-clamped squid giant axons. The time course of block restoration after conditioning depolarization was found to be modulated by membrane electric field, K-channel gating, and external cations. Depolarized holding potentials accelerated block restoration without altering steady-state block levels, suggesting that the voltage dependence of block restoration may be related to K channel gating rather than drug binding per se. In support of this notion, low external calcium concentration, which shifts the voltage dependence of K-channel gating to more negative potentials, also accelerated block restoration. Conversely, the relationship between the rate of block restoration and membrane holding potential was shifted in the depolarizing direction by phloretin, an agent that shifts the dependence of K-channel opening on membrane potential in a similar manner. Modification of K-channel gating also was found to alter the rate of block restoration. Addition of internal zinc or internal treatment with glutaraldehyde slowed the time course of both K-channel activation and aminopyridine block restoration. Aminopyridines also were found to interact in the K channel with external Cs^+, NH_4^+, and Rb^+, each of which slowed aminopyridine block restoration. Our results suggest that aminopyridines enter and occlude K channels, and that the availability of the binding site may be modulated by channel gating such that access is limited by the probability of the channel reaching an intermediate closed state at the resting potential.

INTRODUCTION

Aminopyridines have been found to block the potassium (K) currents in excitable membranes in a manner dependent upon membrane potential and frequency of stimulation, such that the block is relieved by repetitive depolarizing pulses and restored by prolonged repolarization. These observations have been explained on the basis that aminopyridines occlude K channels by binding to closed channels under resting conditions and rapidly dissociating from open channels upon depolarization (Yeh et al., 1976). We have obtained further evidence concerning the mechanism of aminopyridine interaction with closed channels and whether the block is by occlusion.

To reach a site within the channel, drug molecules must travel by either a hydrophilic route associated with the ion pore or a hydrophobic route through the interior of the membrane (Hille, 1977). The hydrophilic pathway appears to be important in the phenomenon of frequency- or use-dependent block by local anesthetics (Hille, 1977), suggesting that these compounds are restricted to interactions with open channels. In a previous study, however, data and model simulations supported the proposition that aminopyridines block closed or resting K channels (Yeh et al., 1976). A possible explanation is that aminopyridines bind preferentially to one or more of the intermediate states that precede opening (Hodgkin and Huxley, 1952; Armstrong, 1975; White and Bezanilla, 1985) such that the time course of onset of aminopyridine block is rate limited by the voltage-dependent probability of a channel progressing through one or more closed states at the holding potential. If such were the case, the onset of block should be accelerated by factors that facilitate channel gating and vice versa. The present experiments were designed to test this hypothesis, the general approach being to correlate changes in aminopyridine blocking kinetics with alterations of K-channel gating. Our results show that depolarization has a dual effect on aminopyridine block; near the resting potential the rate of aminopyridine block is accelerated by depolarization, whereas at strongly depolarized potentials block is removed. The former effect can be modulated by factors that influence channel gating.

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Other K-channel blockers such as Cs^+ and tetraethylammonium (TEA) are thought to occlude the K channel based on the characteristic current-dependent relief of block (Armstrong, 1975). Thus, externally applied Cs^+ appears to bind to sites within the channel lumen, thereby hindering inward movement of K^+ (Hille, 1973; Adelman and French, 1978; Clay and Shlesinger, 1983) and gating (Swenson and Armstrong, 1981; Oxford and Adams, 1981). External Cs^+ also interacts with internally applied long-chain TEA derivatives, such that their removal from the K channel is inhibited by external Cs^+, presumably due to its ability to block inward currents (Armstrong and Bezanilla, 1977). We found that the onset of aminopyridine block was 2–20 times slower in the presence of external cesium, rubidium, or ammonium ions, suggesting that aminopyridines also can enter and occlude the channel lumen.

METHODS AND MATERIALS

Giant axons isolated from *Loligo pealei* obtained at the Marine Biological Laboratory, Woods Hole, Massachusetts, were internally perfused by the roller method of Baker et al. (1961). The axons were mounted in a Plexiglas chamber, perfused externally with chilled artificial seawater (ASW), and voltage clamped by the axial wire method described previously (Oxford, 1981). Briefly, a double axial wire electrode, consisting of a KCl-filled capillary for voltage measurement and platinum black coated platinum wire for passing current, was inserted longitudinally into the axon. The voltage reference electrode was connected to the external solution through an ASW-filled agar capillary positioned just outside the axon near the tip of the internal voltage-sensing electrode. Membrane currents were measured from the bath through a Pt-black electrode held at virtual ground by feedback fron an operational amplifier. This electrode formed the central plate of a three-plate assembly, with the two outer Pt-black plates serving as grounded guards. Usually two-electrode assemblies were used, one on either side of the axon.

The response time (10–90% of a step pulse command) of the voltage-clamp circuit was ~6 μs. Electronic compensation was used to correct for approximately two-thirds of the voltage-control errors arising from series resistance. Linear leakage currents were subtracted electronically from the membrane currents. The axons were held at -80 mV (unless indicated otherwise) and stimulated with 8-ms pulses to a potential of +100 mV. Na-ASW had the following ionic compositions (in millimolars): Na^+, 450; K^+, 10; Ca^{++}, 50; HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid), 5; and Cl^-, 576. In some experiments 220 mM Na^+ was replaced by an equal concentration of either Cs^+, NH_4^+, or Rb^+, and external K^+ was eliminated. External pH ws adjusted to 8.0. Sodium channels were blocked by the addition of tetrodotoxin (1×10^{-6} M) to the ASW. The standard internal solution (SIS) had the following composition (in millimoles per liter): Na^+, 50; K^+, 350; glutamate$^-$, 320; F^-, 50; sucrose, 333; and phosphate buffer, 15. Unless indicated otherwise, the pH was adjusted to 7.3. In experiments in which the pH was varied between 6.3 and 8.55, SIS was buffered with Bis-Tris propane (10 mM, Sigma Chemical Co., St. Louis, MO). Aminopyridines (Aldrich Chemical Co., Milwaukee, WI) were added to the SIS. 4-aminopyridine methiodide (4APMI) was prepared by the method of Poziomek (1963), and had a melting point of 179–180°C. All experiments were performed at a constant temperature of 8–10°C.

Most data were recorded on film and analyzed from enlarged images projected onto graph paper. Some experiments were performed with the aid of an on-line PDP 11/23 computer (Digital Equipment Corp., Maynard, MA), which controlled stimulation and data acquisition at 12- or 14-bit resolution. A two-tailed Student's *t* test for unpaired data was used to determine statistical significance of differences between means ($P < 0.05$).

RESULTS

Voltage and Frequency Dependence of 2, 3-Diaminopyridine Block

The voltage- and frequency-dependent nature of aminopyridine block is illustrated in Fig. 1 (2, 3-diaminopyridine, 0.5 mM). In Fig. 1 *A* repetitive pulses (8 ms duration, +100 mV amplitude, 1 pulse/s) evoked K currents of successively greater amplitude until a constant level was reached after 30 pulses. Increased current amplitude corresponds to decreased block. The time course of block removal (Fig. 1 *E*) is indicated qualitatively by the number of pulses required to reach steady-state.

Block progressively increased during the repolarization that followed a train of stimuli. The time course of the block restoration process was measured by the pulse protocol shown in the upper right of Fig. 1, in which a conditioning train of depolarizing pulses (E_c) was followed by a rest interval of varying duration at the holding potential of −80 mV. The level of block restoration at the end of each interval was determined from K-current amplitude evoked by a single test pulse (E_t, 8 ms, +100 mV). The monotonic decay of test pulse current amplitude (Fig. 1 *F*) gives the time course of block restoration. Typical test pulse currents are shown in Fig. 1, *B–D*. A complete determination required a series of trials, each consisting of paired conditioning and test stimuli with different rest intervals for each pair. Since the conditioning train was always long enough to reach a constant level of block removal, the time interval allowed between successive trials was not critical (usually 30 s was allowed), hence, the determination could be done rapidly. However, restoration time course in the range of 0.5 to 1 s could not be resolved by this method due to the problem of external accumulation of K^+ (Frankenhaeuser and Hodgkin, 1956). Therefore, an alternative pulse protocol (twin pulse) was used in which a single conditioning pulse served to partially remove block and an identical test pulse, applied after a rest interval, was used to assess the amount of block restoration. K accumulation was less of a problem when only a single conditioning pulse was used. However, sufficient time to allow complete block restoration between successive trials was necessary to insure that the response to the conditioning pulse was constant (intervals as long as 3 min were used). Thus, while the method had better temporal resolution it became tedious when block restoration was slow. However, no significant difference was found between the results obtained by these two methods. For example, internal treatment with 0.5 mM 2, 3-diaminopyridine (2, 3-DAP) at pH 7.3 resulted in mean block restoration time constants (τ_{rs}) of 2.9 ±0.3 (mean ± SEM, seven axons) by the twin pulse protocol and 3.8 ± 0.5 (four axons) by the pulse train method.

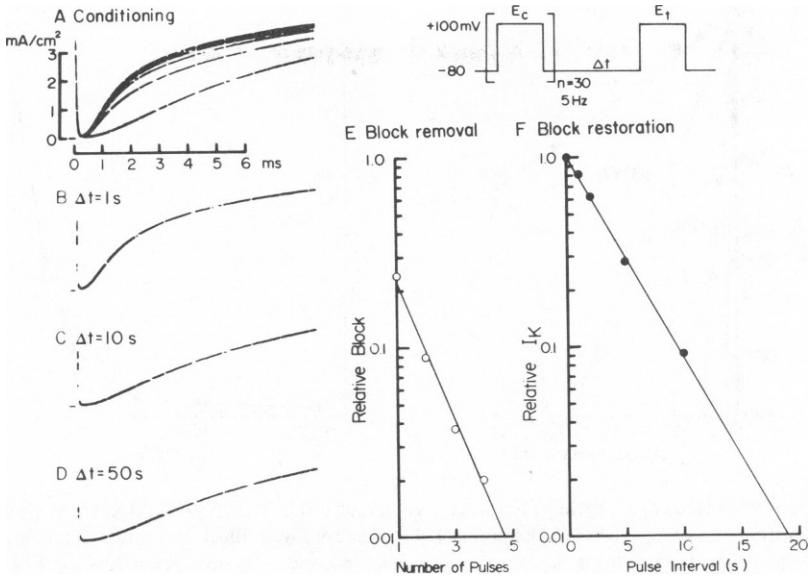

FIGURE 1 Frequency dependent block removal (*A*, *E*) and restoration (*B–D*, *F*) in the presence of 2, 3-DAP (0.5 mM). Inset shows the pulse protocol used to elicit K currents in *A-D*. Thirty conditioning pulses were delivered at a frequency of 5 pulses/s. The first pulse in the train was preceded by a 3-min reset interval and evoked the smallest (i.e., unconditioned) current. Single test pulses were delivered at various intervals (2–20 s) after the conditioning train. Each record in *B–D* was produced by a conditioning train (currents not shown) and a test pulse with a 30-s interval between trials. In *E*, relative block (1-residual current in drug treated axon/drug free current) is plotted as a function of number of pulses in the stimulus train shown in *A*. Current amplitudes were measured 2 ms after the start of each pulse. In *F*, relative current (test pulse/maximum conditioned current) was measured 2 ms after the start of each pulse including those of *B–D*. Solid lines were fit by least squares regression. Internal pH = 7.3. Axon 81A.

Effects of Holding Potential

As shown in Fig. 2 (0.5 mM, 2, 3-DAP), the frequency dependence of aminopyridine block removal appeared to be enhanced when the holding potential was shifted from −40 (*A*) to −100 mV (*D*), as indicated by the number of pulses required to reach a steady state. The effect may be due, in part, to the influence of holding potential on the rate of block restoration during the interval between the individual test pulses of the stimulus train. This appears to be the case for tertiary compounds as shown in Fig. 3 *A* where 2, 3-DAP block restoration time course plotted at various holding potentials appears to be markedly accelerated by depolarizing holding potentials. Similar results were obtained for quaternary compounds as illustrated in Fig. 3 *B* where the relationship between 4-APMI block τ_{rs}

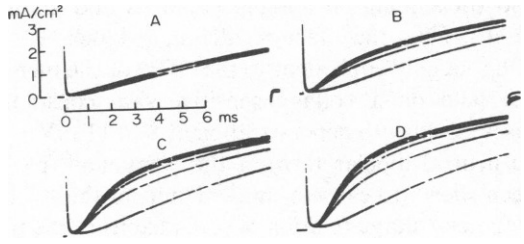

FIGURE 2 Effect of holding potential on frequency dependence of 2, 3-DAP (0.5 mM, 7.3) block. In *A–D* axon was repetitively stimulated (5 pulses/s) to reach a steady level of current (smallest current in each panel was evoked by first pulse). Holding potentials: (*A*) −40, (*B*) −60, (*C*) −80, and (*D*) −100 mV. Axon 725C.

and holding potential was steepest in the potential range −60 to −40 mV. A 14.6 mV (six axons) depolarization caused an *e*-fold decrease in time constant. A similar voltage dependence was observed with other aminopyridines (mV/*e*-fold change): 0.1 mM 4-AP (13.6 0.9, three axons), 0.5 mM 2, 3-DAP (15.0, one axon), and 0.1 mM 3, 4-DAP (14.6, one axon).

This result raises the question of whether the voltage dependence of block restoration reflects a voltage-dependent drug binding reaction or whether another voltage-dependent process such as K-channel gating indirectly influences block by controlling the accessibility of the binding site. If depolarized holding potentials accelerate the drug binding reaction directly, the steady-state level of block should increase as well. However, this was found not to be the case. In Fig. 2 for instance, relative block levels could be determined from the current amplitude at the end of the first pulse in each train compared to drug-free control currents at each holding potential (to compensate for long-term inactivation). Expressed as percent block, the levels were 50.0, 72.5, 72.7, and 70.5, respectively, at holding potentials of −40, −60, −80, and −100 mV. Thus, a shift of −80 to −60 mV caused a fivefold increase in block restoration rate without altering the level of block, whereas one would expect an increase in the rate constant of drug binding to result in about a 20% increase in block. The decrease in block associated with a holding potential of −40 mV may be due to voltage-dependent block removal from open channels.

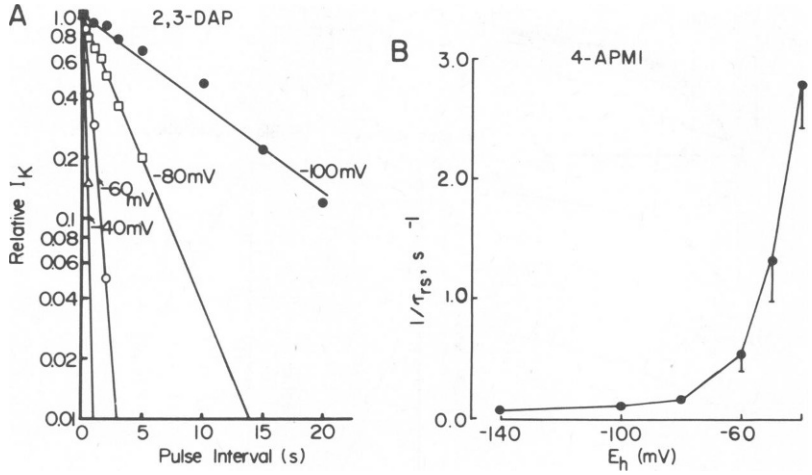

FIGURE 3 Effect of holding potential on aminopyridine block restoration rate. (*A*) 2, 3-DAP (0.5 mM, pH 7.3) block restoration time course plotted semilogarithmically from data obtained using pulse protocol of the inset of Fig. 5. Block restoration time constants (τ_n) of 0.2, 0.6, 3.0, and 10.0 s, respectively, were obtained at holding potentials of −40, −60, −80, and −100 mV. Axon 725C. (*B*) Mean T_n determined in six axons treated with 0.5 mM 4-APMI, $1/\tau_{RS}$ plotted as a function of holding potential.

Similar results were obtained in experiments with other derivatives including 4-AP and 4-APMI (Table I). For both compounds, a shift of the holding potential from −80 to −50 mV resulted in a seven- to ninefold acceleration with no significant change in block level. The lack of correlation between blocking effectiveness and kinetics suggests that the latter is linked to some other voltage-dependent process such as channel activation. This result was unexpected in view of the previous suggestion (Yeh et al., 1976) that aminopyridines preferentially block closed channels and are displaced from open channels by depolarization. We therefore undertook further experiments to test the hypothesis that access of drug molecules to K channels depends on activation gating. If such were the case, we would expect the rate of block restoration to be modulated by both the kinetics and voltage dependence of K-channel activation. Hence, experimental manipulations that shift the voltage dependence of activation to more negative potentials should increase the probability of channel opening at the holding potential, and accelerate block restoration. Conversely, shifting the K-channel activation-voltage relation to more positive potentials should retard block restoration.

TABLE I
EFFECT OF HOLDING POTENTIAL ON AMINOPYRIDINE BLOCK

Aminopyridine	E_H	Block	τ_n
		percent	*s*
4-AP, 0.1 mM	−50	59.0 ± 3.8 (4)	0.7 ± 0.2 (3)
	−80	61.2 ± 3.7 (4)	6.2 ± 0.6 (3)
4-APMI, 0.5 mM	−50	41.0 ± 4.2 (5)	0.9 ± 0.2 (6)
	−80	44.0 ± 3.1 (5)	6.2 ± 0.3 (6)

Effects of Ca^{++} and Phloretin on Aminopyridine Block

Decreasing external Ca^{++} concentration shifts the steady-state voltage dependence of K channel activation to more negative potentials (Frankenhaeuser and Hodgkin, 1957). The effects of this manipulation on 4-AP block are illustrated in Fig. 4. In Fig. 4 *A* a decrease in external Ca^{++} concentration from 50 (solid line) to 5 (broken line) mM was associated with a 1.6- and 2.0-fold decrease in the block restoration time constant at holding potentials of −80 and −50 mV, respectively. As shown in Fig. 4 *B*, the effect of low [Ca^{++}] on block restoration rate was approximately equivalent to a 10 mV depolarization of the holding potential. A comparable effect of low [Ca^{++}] on K-channel gating (−6 mV shift of the midpoint of the steady-state K conductance-voltage relationship) was observed under drug-free control conditions in this axon. Similar results were obtained in experiments using 3, 4-DAP and 4-APMI. A 10-fold decrease in external Ca^{++} concentration (sufficient to shift the K conductance-voltage relationship by an average of −6.7 mV ±1.02, seven axons) caused a decrease in the block restoration time constant of 1.6- to 3.6-fold (depending on holding potential and aminopyridine used). Thus, the effect of calcium on block restoration would be roughly equivalent to the effect of the membrane electric field on a voltage sensitive rate constant that increases *e*-fold with depolarization of 8 to 11 mV.

The neutral dipolar form of the compound, phloretin, has been shown to cause a marked shift of the K channel conductance-voltage relation of squid axons in the positive direction along the voltage axis by as much as 50 mV (Strichartz et al., 1980). This effect is moderately specific to K channels over Na channels and appears to be related to a change in the local dipole field near K-channel

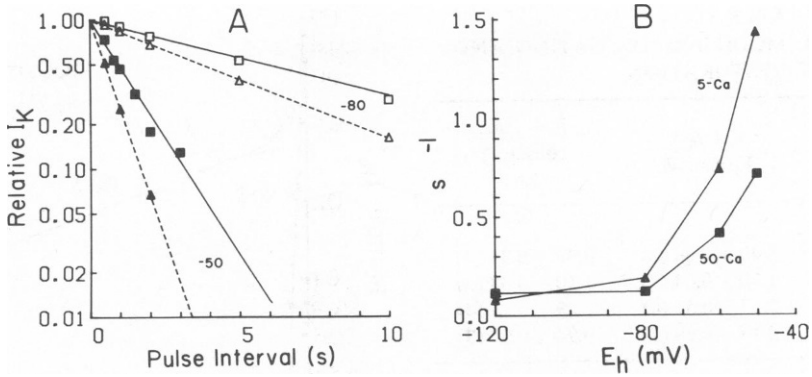

FIGURE 4 Low $[Ca^{++}]_o$ accelerates 4-AP (0.1 mM) block restoration. In *A* block restoration time course is plotted semilogarithmically. Straight lines were fit to data obtained at 50 mM (*solid lines*) and 5 mM (*broken lines*) $[Ca^{++}]$. Time constants of 8.4 and 1.4 s, respectively, describe the 50 mM Ca^{++} data at holding potentials of −80 and −50 mV. Time constants of 5.3 and 0.7 s, respectively, fit the 5 mM Ca^{++} data at −80 and −50 mV. In *B* the rates of block restoration ($1/\tau_n$) in 50 mM (*squares*) and 5 mM (*triangles*) Ca^{++} are plotted as a function of holding potential. Axon 713A, pH 7.3.

activation gates. Block restoration by 3-AP at holding potentials of −40, −60, and −100 mV is illustrated in Fig. 5 *A* in the absence (open symbols) and presence (filled symbols) of 50 μM internal phloretin. The corresponding rate constants are shown as a function of holding potential (Fig. 5 *B*). It can be seen that in the presence of phloretin the block rates were reduced at all potentials, in a manner consistent with a shift in the relationship between block rate and holding potential to more positive membrane potentials. The similarity between this shift and that of the conductance-voltage relation for K channels suggests that 3-AP block is sensitive to channel gating.

Effects of Channel Modifiers

The effects of two agents (internal Zn^{++} and internal F^-) which reversibly modify K channel gating kinetics, and one irreversible modifier (glutaraldehyde) were tested. Each of these chemically dissimilar agents slows K channel gating kinetics. Begenisich and Lynch (1974) have shown that 1 mM Zn^{++} reversibly slows the activation of K channels by a factor of approximately threefold and that the effect is partly relieved by a conditioning hyperpolarization. Table II shows that, in agreement with Begenisich and Lynch (1974), internal Zn^{++} significantly retarded K channel activation and that the effect was potentiated by more

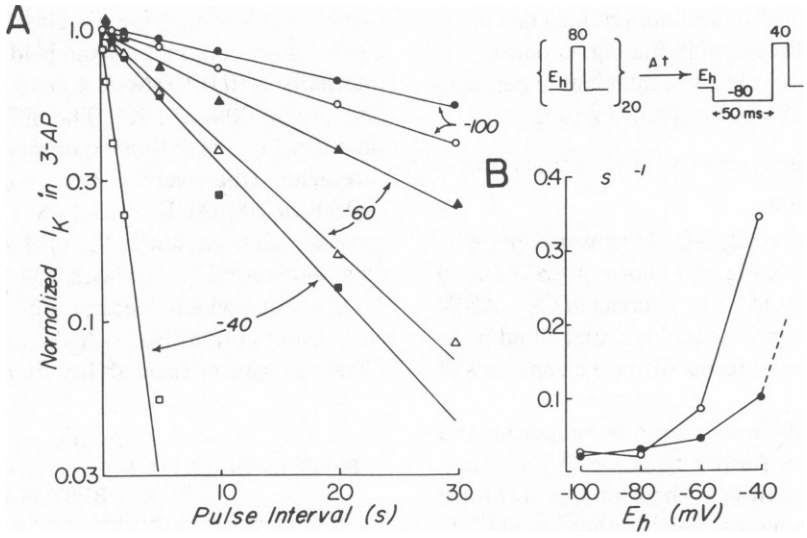

FIGURE 5 Phloretin slows 3-AP block restoration in a voltage-dependent manner. Block restoration rates were determined following the application of 20 conditioning depolarizations to remove block of K currents by 0.5 mM 3-AP. Test depolarizations were applied at varying intervals following the conditioning step and were always preceded by a 50-ms step to −80 mV to normalize the activation kinetics for each holding potential (protocol is shown in the inset). Relative K currents at +40 mV are plotted (*A*) as a function of time for holding potentials of −40 (*squares*), −60 (*triangles*), and −100 mV (*circles*). Measurements were made in the absence (*open symbols*) and presence (*filled symbols*) of 50 μM internal phloretin. The rate constants for block restoration are plotted (*B*) as a function of holding potential for control (*open circles*) and phloretin (*filled circles*) conditions.

TABLE II
EFFECT OF CHANNEL MODIFIERS ON GATING AND BLOCK RESTORATION

Modifier	E_H	Rel. $t_{0.5}$* K channel	Rel. Time constant‡ 4-AP
	mV		
Zn^{++}	−50	3.40 ± 0.60 (2)	2.36 ± 0.35 (4)
	−80	1.70 ± 0.04 (2)	1.50 ± 0.08 (3)
Glutaraldehyde	−80	2.67 ± 0.51 (4)	1.89 ± 0.21 (4)
F^-	−80	6.19 ± 0.69 (2)	0.94 ± 0.07 (3)

*Rel. $t_{0.5}$ = ratio of half rise time of K currents in the presence/absence of gating modifier. No 4-AP.
‡Rel. Time constant = ratio of block restoration time constants in the presence/absence of gating modifier. 4-AP (0.1 MM).

positive holding potentials. Our measurements of half-rise times underestimated the true value because the 16-ms pulses used were too short to achieve steady-state K current in the Zn^{++}-treated axons. Zn^{++} treatment increased aminopyridine block restoration time constants by 1.5- to 2.4-fold, the effect being more pronounced at more positive holding potentials. Similarly, the protein cross-linking reagent, glutaraldehyde, which has been shown to irreversibly slow K current activation (Horn et al., 1980), also slows aminopyridine block restoration by about twofold.

In contrast to the above results, replacement of internal anions with F^- had no effect on aminopyridine block restoration (Table II) even though it caused at least a sixfold slowing of channel activation. However, Adams and Oxford (1983) have shown that the effect of F^- on K currents is partially relieved by aminopyridine concentration as low as 0.04 mM. Presumably the higher concentration of 4-AP used here (0.1 mM) was sufficient to competitively inhibit the effect of F^- on K channel gating.

Interactions Between Aminopyridines and External Cations

The effect of external Cs^+ on 2, 3-DAP (internal pH 6.3) block restoration is shown in Fig. 6. In normal ASW (filled circles) the time constant was 0.8 s, whereas in Cs^+-ASW (open circles) the time course was biphasic and could be fit by the sum of two exponential terms with time constants of 1.1 and 40.3 s.

Similar results were obtained under other experimental conditions. In particular, a similar response to Cs^+ could also be obtained under conditions where the neutral form of aminopyridines predominates. For instance, at pH 8.6, τ_{rs} for 2-AP block (1 mM, one axon; pK_a 6.9, Albert, 1963) increased from 15 to 105 s and for 2, 3-DAP block (0.5 mM, 1 axon; pK_a 7.0, Albert, 1963) τ_{rs} increased from 28 to 86 s. Although accurate measurement of these extremely long time constants is difficult, the qualitative effect of Cs^+ on frequency-dependent block can be described as a decrease in the rate of block restoration.

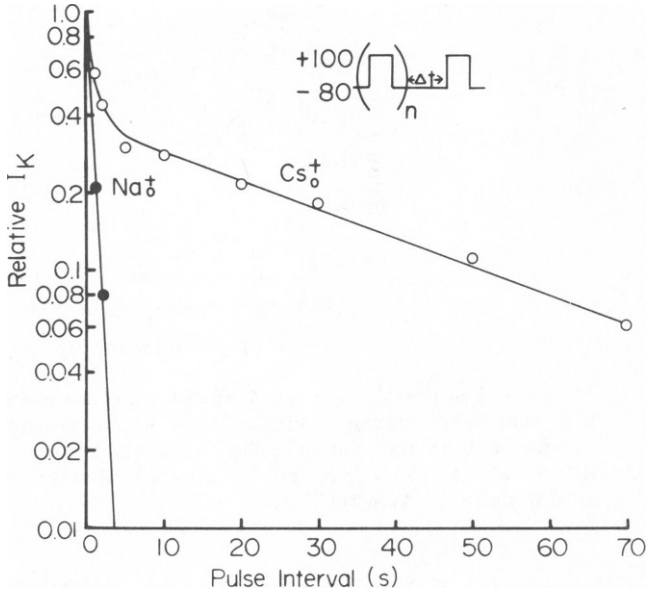

FIGURE 6 Effect of external Cs^+ on 2, 3-DAP (0.5 mM) block restoration. Axon bathed in normal Na-ASW (*filled symbols*) and in ASW containing a mixture of 200 mM Cs^+ + 230 mM Na^+ (*open symbols*). Internal pH 6.3, axon 711A.

A summary of the effects of external cations on 4-AP block restoration is shown in Table III. External Cs^+, Rb^+, and NH_4^+ slowed the rate of 4-AP (0.1 mM) block restoration 20-fold, 3.8-fold, and 2.7-fold, respectively. Block restoration was biphasic in the presence of Cs^+, and monophasic in Rb^+ and NH_4^+.

In contrast, highly permeant ions such as K^+, and highly impermeant ions such as Na^+ and tetramethylammonium (TMA) appear to have no effect on the time course of block restoration. In one axon held at −80 mV and treated internally with 0.1 mM 3, 4-DAP, τ_{rs} was 9.3 s in 10 mM and 13 s in 300 mM K^+. The difference between the two time constants is within experimental error for such measurements (the average time constant for 0.1 mM 3, 4-DAP in 10 mM K^+ was 11.8 ±1.9 s, four axons). In a second axon treated with 0.1 mM 4-AP after total replacement of external sodium with TMA, the time constant was 4.8 s, a value which does not differ significantly from that obtained in normal Na^+−ASW (4.4 ±0.4 s, eight axons). Thus, in terms of their ability to interact with aminopyri-

TABLE III
EFFECTS OF EXTERNAL CATIONS ON 4-AP (0.1 mM) BLOCK RESTORATION

Cation	τ_{rs}
Na^+	4.4 ± 0.4 (8)
TMA^+	4.8 (1)
NH_4^+	11.9 ± 1.2 (4)
Rb^+	16.6 ± 0.9 (5)
Cs^+	80–96 (2)*

*Slowest of two time constants; see Fig. 6.

dines, these cations can be placed in order of effectiveness: $Cs^+ > Rb^+ > NH_4^+ >> Na^+, K^+, TMA^+$.

DISCUSSION

Depolarization drives K channels sequentially through several closed states to reach a single conducting state (Hodgkin and Huxley, 1952; Armstrong, 1975). The unique voltage- and frequency-dependent characteristics of aminopyridine block of K channels have been explained previously by assuming that aminopyridine molecules bind to a site within the closed channel and occlude it without affecting the operation of the gating mechanism. On the other hand, aminopyridines tend to dissociate from conducting channels, the extent of dissociation also being dependent upon membrane depolarization (Yeh et al., 1976; Meves and Pichon, 1977). The present results support a reinterpretation of these observations and are consistent with the idea that aminopyridines can reside within closed channels but must gain access to the blocking site via one or more of the intermediate closed-activated states during channel gating. Access to the binding site is influenced not only by the conformational state of the channel but also by the presence of foreign monovalent cations in a manner correlated with their affinity for sites in the ion pathway.

The main support for this view comes from the dependence of block restoration rates on factors which modulate channel gating. Decreased holding potential and decreased calcium concentration accelerated, whereas the addition of phloretin slowed block restoration rates. The effects of these treatments on K-channel gating offers a unifying explanation for their effects on aminopyridine block restoration. All three manipulations appear to change the electric field strength so as to exert a bias on the probability of voltage-dependent transitions among channel states. This was accomplished by direct changes in transmembrane potential (holding potential changes) or by indirect changes in membrane surface (Ca^{++}) and dipole (phloretin) potentials. In each case an increase in the probability of K-channel opening was associated with faster block restoration, whereas a decreased probability of channel opening resulted in slower block restoration. In addition, slowing the rate of K-channel activation by zinc or glutaraldehyde treatment also resulted in slower block restoration.

The voltage dependence of the block restoration rate may be related to several factors. (*a*) The cationic form of the molecule may pass through some portion of the membrane electric field to reach (and escape) its binding site. (*b*) More than one aminopyridine molecule might be required to block a K channel, thus imparting a greater apparent blocking valence. (*c*) Channel block may derive part of its voltage dependence from K-channel gating. We observed that the voltage dependence of block restoration rate was 13–15 mV/*e*-fold increase in rate. The appropriate voltage dependence for a monovalent cation that experiences the entire membrane electric field is roughly 24 mV/*e*-fold change in rate. Thus, the much steeper voltage dependence of block restoration suggests that either aminopyridine kinetics are governed by a receptor-drug binding stoichiometry greater than 1 or that the voltage dependence of the restoration rate reflects that of the multivalent K-channel gate. The former suggestion appears unlikely as a one-to-one stoichiometry is consistent with aminopyridine dose-response curves (Kirsch and Narahashi, 1978 and 1983) and, as described above, steady-state block is not significantly dependent on holding potential. A determination of the voltage dependence of K-channel activation in squid axons derived from the limiting logarithmic slope of the conductance-voltage curve (Almers and Armstrong, 1980) yields a value of 4.3 mV/*e*-fold increase in g_K. This voltage sensitivity is clearly steeper than that for block restoration rate, but is consistent with the notion that aminopyridines bind to one or more intermediate states of the channel. A recent study of K-channel activation gating (White and Bezanilla, 1985) has indicated that the transitions between the early closed states are the slowest and least voltage dependent. These states, therefore, are the most likely substrates for the aminopyridine binding reaction and the voltage dependence of the steady-state distribution of channels among the closed states of the White-Bezanilla (1985) model might be expected to resemble that of aminopyridine block. We performed a numerical integration (Runge-Kutta method) of this kinetic model at holding potentials of -100 to -40 mV and found that in the range of holding potentials between -80 and -50 mV, depolarization reduced the occupancy of the first closed state with a voltage dependence of 14 mV/*e*-fold change. The close similarity between this voltage dependence and that of the time constant of block restoration is consistent with the notion that the aminopyridine molecule has a higher affinity for the intermediate states of the channel. The drug probably does not bind to the open state since <5% of the channels reach the open state in this potential range. Thus, K-channel activation gating appears to influence the voltage dependence of block in two ways: progression through one or more closed states is necessary to allow access to the aminopyridine binding site and opening of the channel is a prerequisite for voltage-dependent block removal.

We found that the addition of external Cs^+, Rb^+, and NH_4^+ prolonged the time course of block restoration. This phenomenon can be explained by the direct interaction between monovalent cations and aminopyridine molecules occupying sites within the channel lumen. Such interactions could reflect direct competition for such sites or allosteric hindrance of aminopyridine binding. Alternatively, external cations might exert an indirect effect on aminopyridine binding by modulating channel gating so as to prevent access of aminopyridine to its binding site. This

latter explanation, however, is inconsistent with the known effects of cations on K-channel gating. Matteson and Swenson (1986) have shown that K channels close more slowly (i.e., remain open longer) near the holding potential in the presence of external Cs^+, Rb^+, or K^+. This effect would increase the number of channels available for aminopyridine block and, as discussed above, speed block restoration. Thus, an indirect effect of cations on gating is in the wrong direction to explain our results.

Experimental and theoretical treatment of ion passage through K channels have suggested multiple ion binding sites (e.g., Hille and Schwarz, 1978). Occupancy of a binding site in the K channel by Cs^+, for example, might result in exclusion of aminopyridine cations and reduce the rate of block. The order of effectiveness with which external ions retard the rate of aminopyridine binding is not related to permeability since two relatively impermeant ions, Cs^+ and TMA^+, occupied the two extremes of effectiveness. Instead, effectiveness may be related to the strength of binding of the cation to the channel or the location of the cation binding sites relative to the aminopyridine site. Assuming that the cation binding site is the same for all ions, a relationship between the effect of retarding aminopyridine binding and the ability of the cation to resist dissociation from the K channel may exist. Thus, our data suggest that Cs^+ should be most tightly bound to the channel followed by Rb^+ and NH_4^+. This conclusion is in agreement with recent determinations of the apparent affinities of these cations for binding sites in the K-channel ion pathway (Matteson and Swenson, 1986) based on instantaneous current-voltage relationship and multi-occupancy barrier models. Their calculations reveal that the strength of ion binding to a site located within the intracellular end of the pore exhibits the following order: $Cs^+ > Rb^+ > K^+ > NH_4^+$.

In summary, the kinetic model originally proposed by Yeh et al. (1976) was based on the assumption that the slow rate of block restoration represents the intrinsic association rate of aminopyridines with binding sites within the closed channels. The present results suggest that access to this binding site may be limited by voltage-dependent gating transitions, perhaps between closed states. Therefore, the rate of block restoration is modulated by the same factors that influence the probability of the channel opening at the holding potential.

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