

# Dynamics of Aminopyridine Block of Potassium Channels in Squid Axon Membrane

J. Z. YEH, G. S. OXFORD, C. H. WU, and T. NARAHASHI

From the Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710. Dr. Oxford's present address is the Department of Physiology, University of North Carolina, School of Medicine, Chapel Hill, North Carolina 27514.

**ABSTRACT** Aminopyridines (2-AP, 3-AP, and 4-AP) selectively block K channels of squid axon membranes in a manner dependent upon the membrane potential and the duration and frequency of voltage clamp pulses. They are effective when applied to either the internal or the external membrane surface. The steady-state block of K channels by aminopyridines is more complete for low depolarizations, and is gradually relieved at higher depolarizations. The K current in the presence of aminopyridines rises more slowly than in control, the change being more conspicuous in 3-AP and 4-AP than in 2-AP. Repetitive pulsing relieves the block in a manner dependent upon the duration and interval of pulses. The recovery from block during a given test pulse is enhanced by increasing the duration of a conditioning depolarizing prepulse. The time constant for this recovery is in the range of 10–20 ms in 3-AP and 4-AP, and shorter in 2-AP. Twin pulse experiments with variable pulse intervals have revealed that the time course for re-establishment of block is much slower in 3-AP and 4-AP than in 2-AP. These results suggest that 2-AP interacts with the K channel more rapidly than 3-AP and 4-AP. The more rapid interaction of 2-AP with K channels is reflected in the kinetics of K current which is faster than that observed in 3-AP or 4-AP, and in the pattern of frequency-dependent block which is different from that in 3-AP or 4-AP. The experimental observations are not satisfactorily described by alterations of Hodgkin-Huxley *n*-type gating units. Rather, the data are consistent with a simple binding scheme incorporating no changes in gating kinetics which conceives of aminopyridine molecules binding to closed K channels and being released from open channels in a voltage-dependent manner.

## INTRODUCTION

Ionic channels in excitable membranes are subject to various pharmacological, enzymatic, and chemical modifications. The selective influence of such manipulations on ionic conductances is a strong argument for separate channels for the movement of sodium and potassium ions. Because of the specific and selective features, certain agents have become indispensable tools for studying the behavior of ionic channels. Tetrodotoxin (TTX) which selectively blocks sodium channels and tetraethylammonium ion (TEA) which blocks potassium channels are two such examples.

4-Aminopyridine has been reported to block selectively the potassium channel in axons of the cockroach (Pelhate and Pichon, 1974), squid (Meves and Pichon, 1975; Yeh et al., 1976a), *Myxicola* (Schauf et al., 1976), and frog node of Ranvier (Wagner and Ulbricht, 1975). In the squid axon and frog node of Ranvier the aminopyridine block of potassium channels is not a simple reduction of conductance, but exhibits voltage-, time-, and frequency-dependent characteristics.

The main objective of this investigation is to characterize the dynamics of interaction of aminopyridines with the K channel. On the basis of the kinetic analysis of the interaction to be presented and two key assumptions, we formulate a model which can account for many of the experimental observations. The first assumption is that the gating mechanism of K channels is not significantly affected by aminopyridines, and the second is that aminopyridine-bound K channels cannot pass K ions even when their activation gates are open. A preliminary report of this work has been presented at the 20th annual meeting of the Biophysical Society (Yeh et al., 1976b).

#### METHODS

Experiments were performed on giant axons isolated from *Loligo pealei* obtained at the Marine Biological Laboratory, Woods Hole, Mass. Both intact axons and axons internally perfused by the roller technique of Baker et al. (1961) were used in these studies. Cleaned axons were mounted in a Plexiglas chamber designed for voltage clamping by conventional techniques described previously (Wu and Narahashi, 1973). Briefly, a "piggyback" double axial electrode assembly was inserted into the axon for measurement and control of membrane potential. Membrane current was measured by the virtual ground of an operational amplifier from a Pt-black electrode situated within a region of the chamber electrically guarded to maximize radial current flow. The response time of the clamp was  $\sim 6 \mu\text{s}$  (10–90% of step pulse command). Feedback compensation was used in all experiments to compensate for errors arising from approx. two-thirds of the measured 3–4  $\Omega/\text{cm}^2$  of series resistance. Holding potentials were  $-80 \text{ mV}$  for internal perfused axons and  $-70 \text{ mV}$  for intact axons.

The axons were perfused externally with artificial seawater (ASW) containing ions in the following concentrations (mM):  $\text{Na}^+$ , 450;  $\text{K}^+$ , 10;  $\text{Ca}^{++}$ , 50; HEPES buffer, 5;  $\text{Cl}^-$ , 576. In several experiments an external bathing medium with elevated potassium concentration was used by equimolar replacement of 340 mM Na with K. External pH was adjusted to 8.0 in both cases. The standard internal solution (SIS) was composed as follows (mM):  $\text{Na}^+$ , 50;  $\text{K}^+$ , 350; glutamate $^-$ , 320;  $\text{F}^-$ , 50; sucrose, 333; phosphate buffer, 15; and was adjusted to a pH of 7.3. All experiments were performed at a constant temperature of 8°–10°C.

Most experiments were performed with 300 nM tetrodotoxin in the external bathing medium to eliminate current contributed by sodium channels. 2-, 3-, and 4-aminopyridine were obtained from Aldrich Chemical Company (Milwaukee, Wis.) and used without further purification.

#### *Data Analysis, Terminology, and Computations*

Oscilloscope records of membrane current and voltage were captured on 35-mm film and analyzed by hand with the aid of a programmable calculator. Comparisons of data obtained with the Hodgkin-Huxley (H-H) model for potassium conductance (Hodgkin and Huxley, 1952) were performed by using the empirical formulae for  $\alpha_n$  and  $\beta_n$  given by Palti (1971) and assuming a  $Q_{10} = 3$ . Actual simulations of voltage clamp experiments were performed on an HP9821 programmable calculator with 9864 digital plotter output

(Hewlett-Packard Co., Cupertino, Calif.) by use of a kinetic equivalent of the H-H model (Armstrong, 1969) as described in the Discussion.

The terminology of "gate" or "gating" as adopted in this paper implies no physical scheme of ionic channels but merely refers to the normal processes by which K channels allow ions to pass across the membrane whatever the mechanism may be. The three analogs of aminopyridine will be referred to collectively as *n*-AP in situations where differences in their effects are not readily distinguishable.

## RESULTS

Aminopyridines (*n*-AP) selectively suppress potassium currents in the squid giant axon when applied to either internal or external membrane surfaces (Fig. 1). Sodium currents remain unaffected by *n*-AP treatment. Potassium tail cur-

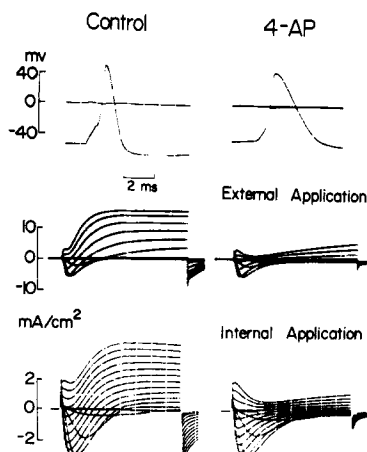


FIGURE 1. Effects of 4-aminopyridine on squid giant axons. Upper records are membrane action potentials, and middle records are clamp families before and after external application of 1 mM 4-AP. Lower records are clamp families from another axon before and after internal application of 1 mM 4-AP. Note only slight prolongation of action potential despite a dramatic reduction of K current.

rents at the end of 8-ms depolarizing steps are reduced due to both a suppression of potassium conductance and a resultant reduction in  $K^+$  ion accumulation in a periaxonal space. The block of  $I_K$  is dramatic but not complete for larger depolarizations. Despite the reduction in  $I_K$  the action potential duration is only slightly prolonged by *n*-AP. In the presence of *n*-AP the potassium current rises more slowly than in control (Yeh et al., 1976a) reaching to a steady-state level only after tens of milliseconds. Therefore, the suppression of K currents by *n*-AP was evaluated at steady-state levels as well as at early times. Fig. 2 demonstrates that the inhibition of  $I_K$  at a given potential is less at the end of a 70-ms pulse than after 8-ms (see also Table I).

This duration-dependent block is shared by all three aminopyridines; however, there are some differences in potency among them. Fig. 3 illustrates the more rapid rise of  $I_K$  in 2-AP (*b*) than in 3-AP (*a*). The upper trace in each case is a control  $I_K$  in ASW alone. The curves in Fig. 3c and d are simulations of the data with a kinetic model to be described later. Differences in potency among the

aminopyridines for reducing  $I_K$  are summarized in Table I which clearly indicates that 2-AP is the least potent in inhibiting K currents in squid axons, whereas 3- and 4-AP exert about the same effect.

The concentration dependence of *n*-AP was difficult to evaluate because of the effect being complicated by the membrane potential, and the duration and frequency of pulses. Table I shows that 30 mM 2-AP is only slightly more

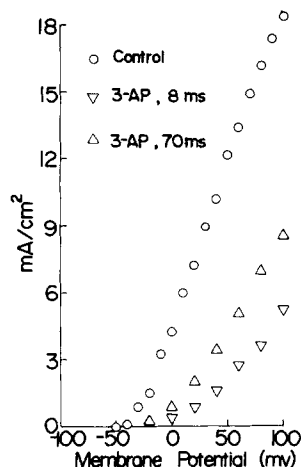


FIGURE 2. Time-dependent block of K current by 3-AP. Current-voltage relations of an axon were measured in the presence of 300 nM TTX externally.  $\circ$ , current measured at the end of 8-ms pulse before application of 3-AP;  $\nabla$  and  $\triangle$ , currents measured at the end of 8 ms and 70 ms, respectively, after external perfusion with 1 mM 3-AP.

TABLE I  
EFFECT OF AMINOPYRIDINES ON POTASSIUM CURRENT MEASURED AT 0 AND 100 MV

<i>n</i> -AP	Concn mM	Percent inhibition* measured at			
		8 ms		70 ms	
		0 mV	100 mV	0 mV	100 mV
2-AP	1	74.8±2.72	21.6±2.92		
	30	81.0±1.00	62.8±2.85		
3-AP	1	93.8±2.20	73.3±1.70	80.4±1.60	50.3±3.25
	30	99.5±0.05	79.2±2.00	92.0	66.5
4-AP	0.03	70.0±3.79	55.3±4.06		
	0.10	88.3±3.18	59.0±8.54		
	0.30	90.0	82.0		
	1	96.2±1.92	74.7±2.29		
	10	94.0	88.0		

\* Mean ± SEM (*n* = 3).

effective than 1 mM when the effect is evaluated at 0 mV and is three times more effective when evaluated at 100 mV. The effect of 3-AP is almost saturated at 1 mM.

#### *Voltage-Dependent Block of $I_K$*

Aminopyridines suppress the potassium current in a manner dependent upon membrane potential. In Fig. 4 the ratio of  $I_K$  in 3-AP to  $I_K$  in ASW without 3-AP is plotted as a function of membrane potential.

The K currents were measured in two ways. The triangles represent measurements of steady-state  $I_K$  at the end of the first of two 70-ms pulses separated by a 1-s interval. The circles are measurements taken at 5 ms after the onset of the

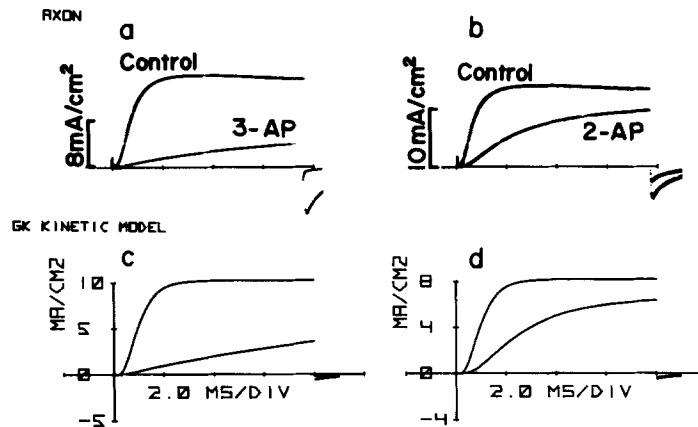


FIGURE 3. Patterns of K current in 2- and 3-AP. Membrane currents during a clamp pulse to +100 mV before and after external application of 1 mM 3-AP (a) or 1 mM 2-AP (b). ASW contained 300 nM TTX in each case. Simulation of current patterns in 3-AP (c) and 2-AP (d) with kinetic model described in Discussion. In control  $k = 0$ ,  $l = 0$ , and  $\tau_r = 0$ . In 3-AP  $k = 0.06 \text{ ms}^{-1}$ ,  $l = 0.01 \text{ ms}^{-1}$ , and  $\tau_{r1} = 3 \text{ s}$ , and  $\tau_{r2} = 27 \text{ s}$ . In 2-AP  $k = 0.3 \text{ ms}^{-1}$ ,  $l = 0.1 \text{ ms}^{-1}$ , and  $\tau_r = 0.8 \text{ s}$ . At rest (-80 mV)  $U' = 0.95$  in both cases.

second pulse of fixed amplitude (+100 mV). This latter procedure is to minimize errors due to  $K^+$  accumulation in the periaxonal space which decays with a time constant of  $\sim 30 \text{ ms}$  (Frankenhaeuser and Hodgkin, 1956), much shorter than that for re-establishment of  $n$ -AP block after a long pulse (see below). The voltage dependence determined in this manner is approximately linear for the voltage range studied and very little affected by the choice of measurement procedure.

#### *Frequency-Dependent Recovery from $n$ -AP Block*

The inhibition of potassium currents by  $n$ -AP is markedly dependent upon the frequency of stimulation (Yeh et al., 1976a). A progressive recovery of  $I_K$  occurs during repetitive applications of depolarizing steps which is dependent upon the pulse frequency. As seen in Fig. 5 the removal of block in 3-AP by successive

pulses is greater for increasing pulse frequencies, saturating at approximately 2 Hz. Fig. 6 illustrates the current patterns obtained in 3- and 2-AP during twin depolarizing pulses to +100 mV separated by a 1-s interval. The rise of  $I_K$  is markedly accelerated during the second pulse in 3-AP (*a*) but approaches the

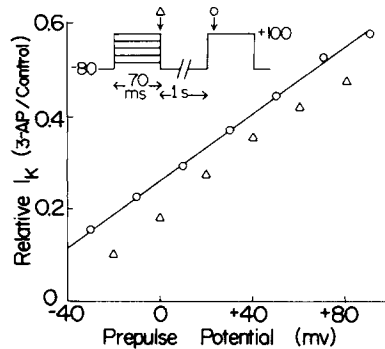


FIGURE 4. Voltage-dependent block of K channels by aminopyridines. Two consecutive 70-ms voltage steps separated by a 1-s interval were applied to an axon bathed in ASW and then in 3-AP. The amplitude of the first or conditioning pulse is varied while the second pulse is fixed at +100 mV. K current measured in 3-AP relative to steady-state (8 ms) values in ASW is plotted as a function of the conditioning pulse amplitude. Current was measured either at the end of the 70-ms prepulse ( $\Delta$ ) or after 5 ms of the test pulse ( $\circ$ ). Solid line represents a least-squares regression fit to the data points obtained during the test pulse.

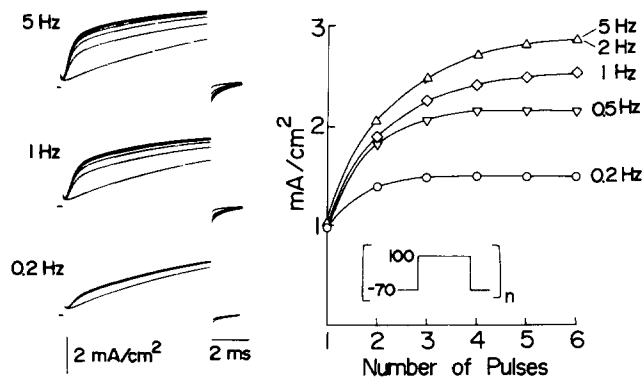


FIGURE 5. Frequency-dependent recovery from aminopyridine block. Potassium currents produced by six consecutive pulses to +100 mV at the indicated frequencies after external application of 1 mM 4-AP. A 3-min rest period was allowed between each trial frequency. Increases in current during consecutive pulses at several frequencies are shown to the right of the current records. Data points were measured after 2 ms of each pulse. Curves are drawn by eye through the points.

same steady-state level (*b*). The effect of the conditioning pulse is less dramatic in 2-AP (*c*), presumably because the removal of block was essentially completed during the first pulse. Fig. 6*d-f* depicts simulations of the twin pulse experiments with the kinetic model described in the Discussion.

In order to examine further the frequency dependence of block, two series of experiments were performed. In the first series the time course for re-establishment of channel block after removal of inhibition by a single pulse was determined. Twin depolarizing pulses to +100 mV were applied and the interval between the first and second pulses varied. A 3-min rest period was allowed between each pair of pulses to assure uniform conditions for each trial. Fig. 7

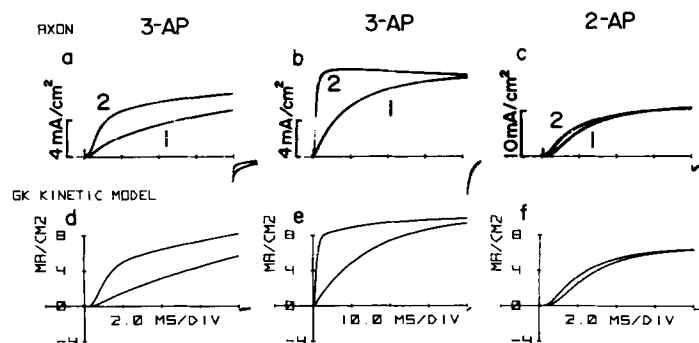


FIGURE 6. Frequency-dependent block of K channels by aminopyridines. Current patterns obtained for two consecutive depolarizing steps to +100 mV separated by a 1-s interval in 1 mM 3-AP (*a, b*) and 1 mM 2-AP (*c*). Simulations of each current pattern by the kinetic model are shown (*d-f*) below the respective experimental pattern. The model parameters used in these simulations are identical to those used in Fig. 3 corresponding to 2- and 3-AP. Time scales for *a-c* are the same as for *d-f*, respectively. The diminished frequency dependence in 2-AP reflects the more rapid interaction of 2-AP molecules with the binding site ( $k = 0.3 \text{ ms}^{-1}$ ,  $l = 0.1 \text{ ms}^{-1}$ ,  $\tau = 0.8 \text{ s}$ ).

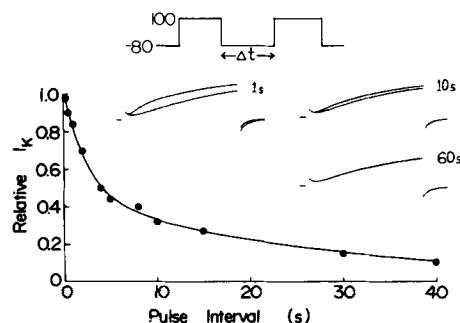


FIGURE 7. Re-establishment of K channel block by aminopyridines. Two consecutive pulses to +100 mV separated by a variable time interval were applied to an axon bathed in 1 mM 3-AP and 300 nM TTX. The ratio of the increase in  $I_K$  during the second pulse to the maximum observed increase is plotted as a function of pulse interval. Currents were measured at 0.5 ms of each pulse. Sample current patterns for intervals of 1, 10, and 60 s are shown in the insets. Block proceeds with two time constants of  $\sim 3 \text{ s}$  and  $\sim 27 \text{ s}$ . Solid line is fit to the data points by the equation  $I/I_{\max} = 0.55 \exp(-t/3) + 0.45 \exp(-t/27)$ , where  $t = \text{time in seconds}$ .

illustrates the results of this type of experiment. The normalized difference in  $I_K$  between the first and second pulses was measured at 0.5 ms after each step and plotted as a function of the interval between pulses representing the time course of re-establishment of inhibition. In the case of 3-AP the time course exhibits two components, a fast time constant (3 s) and a slower one (27 s). In contrast, the re-establishment of block in 2-AP had a much faster time course. Superimposable K current traces could be produced in 2-AP when the two pulses were separated by an interval of only 2 s. Consequently, the time constant for re-establishment of block in 2-AP is estimated to be less than 1 s.

Re-establishment of block is more rapid as the concentrations of *n*-AP are increased. For example, in the presence of 30 mM *n*-AP the potassium currents associated with the twin pulses could be reproduced for pulse intervals as short as 2 s in the case of 3- or 4-AP and only 1 s in 2-AP.

#### *Time Course of Removal of n-AP Block*

The second series of experiments was designed to examine the time course of recovery from block during multiple pulses. The pulse schedule again used twin depolarizing pulses to +100 mV in this case with a fixed 1-s pulse interval but with a variable first pulse duration. In Fig. 8 the recovery of  $I_K$  is plotted as the current measured at 3 ms of the second pulse vs. the duration of the first or conditioning pulse. The recovery from 3-AP block usually followed a single exponential time course, with a time constant of approximately 10–20 ms. This can perhaps be visualized as representing the time constant governing the interaction of 3- or 4-AP with a site within the open K channel. While the opening of the K channel gating mechanism is probably required for the pulse-dependent recovery of  $I_K$ , the rate-limiting step appears to be the dissociation of the *n*-AP molecule from the open channel. This is apparent, as the time constant for recovery from block is approximately 10 times longer than that for normal activation of K channels. To the right of the recovery plot in Fig. 8 are typical records used to construct the plot (upper traces) and the results of simulating the same protocol on the kinetic model (lower curves).

#### *Steady-State Inhibition Is Less in High External K*

Experiments in high external potassium have shown *n*-AP to suppress currents flowing in either direction through K channels (Yeh et al., 1976*a*). In 340 mM K seawater, the resting membrane potential is approximately 0 mV and external application of 1 mM 2-, 3-, or 4-AP produced no appreciable change. Therefore, the membrane potential could be clamped at zero without polarizing the current-passing electrode. Step depolarizations to various potentials were applied and quasi-instantaneous currents were measured. The suppression of K currents by 3-AP under these conditions is seen in Fig. 9. A linear I-V relation was found both in the control and *n*-AP axons (Yeh et al., 1976*a*). The decrease in slope of the I-V relation thus represents the steady-state inhibition of K conductance at 0 mV by *n*-AP. The percentage of inhibition was not significantly different among these compounds, ranging from 22% to 28%. Important to note, however, is that the suppression is much smaller than observed at 0 mV (8 ms) for axons in normal K seawater (see Table I). This discrepancy may be



ascribed to differences in the number of open channels at the holding potentials used in each case, 0 mV in high K and -80 mV in normal ASW. Also quantitative differences in the degree of steady-state block are influenced by the interactions between K ions and *n*-AP molecules, inactivation of  $g_K$ , and K ion accumu-

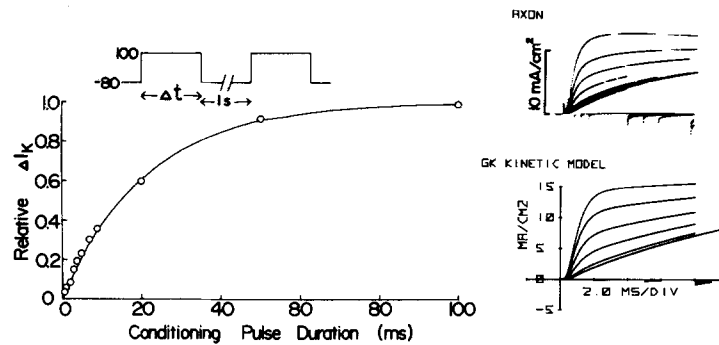


FIGURE 8. Pulse-dependent recovery from aminopyridine block. Two consecutive pulses to +100 mV with 1-s interval were applied to an axon bathed in 1 mM 3-AP and 300 nM TTX. The duration of the first pulse was varied and a 3-min rest period allowed between pulse pairs. K current was measured at 3 ms during the second pulse. The ratio of  $I_K$  to the maximum obtainable  $I_K$  is plotted as a function of the first pulse duration. Solid line represents a single exponential fit to the data points with a time constant of 21 ms. Typical pattern used to extract such data is shown to the right with its simulation by the kinetic model. Conditioning pulse durations in the stimulation were 1, 2, 5, 10, 20, and 50 ms, consecutively. Model parameters are again identical to those used in Fig. 3 ( $k = 0.06 \text{ ms}^{-1}$ ,  $l = 0.01 \text{ ms}^{-1}$ ,  $\tau_{r1} = 3 \text{ s}$ , and  $\tau_{r2} = 27 \text{ s}$ ).

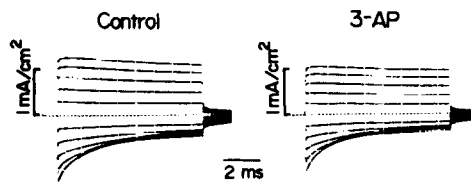


FIGURE 9. Nonrectifying block of  $I_K$  by 3-AP. 3-AP (1 mM) was applied externally to an intact axon bathed in high-potassium seawater (340 mM  $K^+$ ). The axon was voltage clamped at the resting potential (0 mV) between depolarizing and hyperpolarizing voltage steps applied every 5 s. Note that both inward and outward  $I_K$  were reduced, but by a smaller amount than in normal K seawater.

lation. These factors will be discussed later. At high depolarization in elevated external  $[K^+]$ , currents undergo a time-dependent recovery reaching control levels in 2- and 4-AP after  $\sim 8 \text{ ms}$  (Yeh et al., 1976a). Such a phenomenon is not observed with 3-AP (Fig. 9). This may be related to the charge state of the molecule, as 3-AP ( $pK_a = 6.03$ ) exists almost exclusively in its neutral form at pH 8.

## DISCUSSION

The interaction of aminopyridines with potassium channels of the squid axon membrane has been shown to exhibit a complicated pattern of voltage, time, and frequency dependence. The steady-state reduction of  $I_K$  by  $n$ -AP is partially relieved at high depolarizations. In addition, the remaining  $I_K$  exhibits much slower apparent turn-on kinetics. While such phenomena seem to suggest an alteration of the conformational freedom of K channel gating components, further consideration of the data reveals inconsistency with such an interpretation. Alternatively, it is suggested that, at the level of a single channel, the effects of aminopyridines are not intimately related to the molecules responsible for the opening and closing of the K ion pathway.

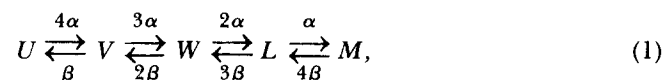
Potassium currents obtained at the end of 8-ms pulses in  $n$ -AP were converted to chord conductance values ( $G_K$ ) as a function of membrane potential. Amplitude scaling of the  $G_K$ -voltage relation in combination with shifts along the voltage axis results in inadequate fits of the experimental values in  $n$ -AP to steady-state  $G_K$  in control. This implies that neither a reduction in the maximum conductance of a single K channel nor a shift of the "set point" of the gating voltage sensor can account for the observed effects.

In addition, if one assumes the Hodgkin-Huxley  $n$  parameter adequately to represent the operation of potassium channels, it is seen that numerous alterations of both the first order rate constants  $\alpha_n$  and  $\beta_n$  and the power function exponent (Cole and Moore, 1960) fail to match adequately the kinetic pattern of  $I_K$  in  $n$ -AP (for example, see Fig. 10). This would seem to rule out a major change in the first order transition of  $n$ -type gating units by  $n$ -AP, if one assumes that these or similarly behaving components are involved in normal K channel gating.

One might, however, conceive of such alterations occurring in only a fraction of the total channel population and being disguised in a functional summation with normally operating channels. This possibility was examined by computer simulation using various combinations of normal channels summed with channels having altered  $n$  parameters to yield a total  $G_K$ . The progressive increase in the fractional population of K channels having been either slowed by a factor of 10 or delayed by increasing the power function exponent results in a clearly distinguishable kinetic component (see Fig. 10*b*) which has no counterpart in actual experimental data (for example, Fig. 3). Restricting the simulated change in  $\alpha_n$  and  $\beta_n$  to five times results in a more monotonic curve, but fails to describe adequately the experimentally observed slowing of  $I_K$ . The lack of success with this approach thus prompts us to suggest an alternative mechanism of  $n$ -AP action which is independent of the gating properties of K channels irrespective of their origin.

*G<sub>K</sub> Kinetic Model*

The kinetics of normal K channel gating can be described by a linear reaction sequence as follows:



where  $\alpha$  and  $\beta$  are defined by the empirical formulae of Hodgkin and Huxley (1952) for  $\alpha_n$  and  $\beta_n$ , respectively, and  $M$  represents the open or conducting state. This reaction scheme is equivalent to transitions of H-H  $n$ -type units of normal K channels between fully closed ( $U$ ) and open ( $M$ ) states (Armstrong, 1969) resulting in kinetics identical to  $\bar{g}_K n^4 (E_m - E_K)$  of the H-H model where  $E_m$  represents membrane potential and  $E_K$  the K equilibrium potential.

Aminopyridine molecules are assumed to interact with K channels by binding to a site(s) within the channel. Channels in which  $n$ -AP molecules occupy the binding site are occluded and thus cannot conduct  $K^+$  ions regardless of the state

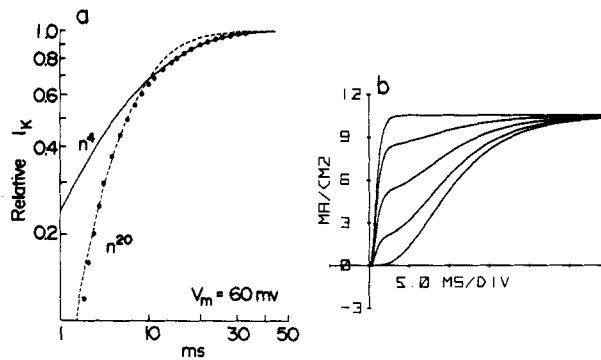
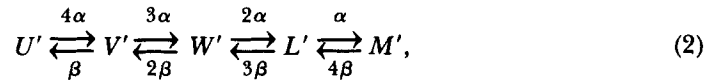


FIGURE 10. Modifications of K channel gating do not describe aminopyridine effects. (a) Data points represent normalized  $I_K$  after external application of 1 mM 4-AP plotted as a function of time in log-log format. Solid and dashed lines are the best fit to the data points of the Hodgkin-Huxley  $n$  parameter raised to powers of 4 and 20, respectively. The fit in each extreme is inadequate to describe the data points. (b) Simulation of K currents by a kinetic model incorporating two linear reaction schemes equivalent to the H-H model as described by Armstrong (1969). Normal rate constants  $\alpha_n$  and  $\beta_n$  were used in one set of reactions while the other set of reactions used rate constants slowed by a factor of 10. Equations describing each set were solved simultaneously and independently and the conducting state values of each set were summed and multiplied by the driving force to yield total current. The fraction of total conductance assigned to the slower population in each curve (lower to upper) is 1.0, 0.8, 0.5, 0.2, and 0.

of the activation gating machinery. The gating of  $n$ -AP bound channels is depicted by the kinetic sequence:

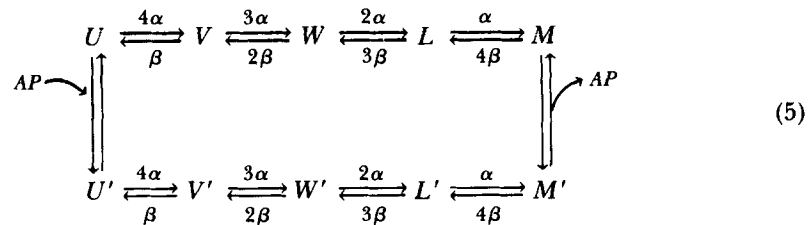


where transitions between states are assumed to be unaffected by  $n$ -AP binding and are thus identical to normal unbound channels. The failure of the  $M'$  state to conduct  $K^+$  ions is assumed to result from occlusion of channels in this state by  $n$ -AP molecules rather than from interference with normal gating processes.

The distribution of total channel population between normal (reaction 1) and AP-bound (reaction 2) conditions is governed by binding and release reaction schemes:



where  $AP$  represents aminopyridine concentration,  $M' = M \cdot AP$ , and  $U' = U \cdot AP$ . These reactions are incorporated into the two linear reaction sequences for normal and bound channels to describe the total channel population as the following scheme:



In this view aminopyridine molecules bind in a dose-dependent manner at a site(s) within the resting (nonconducting) potassium channel ( $U \rightarrow U'$ ). At rest (holding potential =  $-80$  mV) all channels are assumed to reside in either  $U$  or  $U'$  with  $\sim 95\%$  in  $U'$  at steady state with  $1$  mM  $n$ -AP present. Upon depolarization both normal and AP-bound channels can undergo identical transitions from closed to open states with H-H  $n$ -type kinetics. In the open state AP-bound (occluded) channels may become conducting by release of  $n$ -AP from the binding site (reaction 4). The kinetics of the release reaction results in a slow turn-on of  $I_K$  as observed experimentally. Parameters of the model pertaining to binding and release of  $n$ -AP are derived from the experimental data in the following manner.

The exact kinetic parameters of the binding scheme are extremely difficult, if not impossible, to determine experimentally. Standard measurements of the rate of onset of drug effects are complicated by the pulse-dependent feature of the block, as well as by diffusion barriers presented by periaxonal structures. Whereas it is not possible to determine forward and reverse rate constants for reaction 3 from available data, time constants associated with the block process can be estimated from the time course of re-establishment of  $n$ -AP block (Fig. 7). As shown previously, the data are best described by a two time constant expression, thus reaction 3 may actually represent a two-step process. Although no exact origin of these two time constants is known, we speculate that the faster one ( $\tau = 3$  s) may be related to the AP-binding site interaction while the slower one ( $\tau = 27$  s) perhaps reflects redistribution of  $n$ -AP molecules near the channel. Rather than making arbitrary assumptions concerning the formal kinetic sequence between  $U$  and  $U'$ , we chose to describe the channel distribution by an analytical expression. At the holding potential ( $-80$  mV) the total channel population was normalized to one and distributed initially as  $U = 0.05$  and  $U' = 0.95$  in  $1$  mM  $n$ -AP. After a simulated depolarizing step and subsequent redistribution between normal and bound populations due to reaction 4 ( $M' \rightarrow M$ ), the distribution between  $U:U'$  was set equal to  $(U + V + W + L +$

$M):(U' + V' + W' + L' + M')$  at the end of the pulse (taking advantage of the fact that normal gating kinetics is much faster than reaction 4). The new initial distribution for the next pulse after an interpulse interval  $\Delta t$  was determined according to:

$$\frac{U}{U'} = A \exp(-\Delta t/\tau_1) + B \exp(-\Delta t/\tau_2), \quad (6)$$

where  $A = 0.55$ ,  $B = 0.45$ ,  $\tau_1 = 3$  s (3- or 4-AP) or 0.8 s (2-AP), and  $\tau_2 = 27$  s. This procedure allowed satisfactory simulation of frequency-dependent phenomena (Figs. 6 and 8).

The removal of block was a linear function of the membrane potential beyond the level (+20 mV) where potassium channels are fully open (Fig. 4). Since the opening of the K channel is a prerequisite for the release of *n*-AP from the channel and also since the latter process is much slower than the former, it is not surprising to find that the removal of *n*-AP block does not saturate at +20 mV. To account for the voltage-dependent recovery observed experimentally (Fig. 4), the release rate constant was given the empirical form  $K = aE_m + b$ , where  $a$  and  $b$  are constants for a given *n*-AP concentration. This results in effectively weaker binding at larger depolarizations (see Fig. 4). In 1 mM 3- or 4-AP  $a = 0.5$  ms<sup>-1</sup> V<sup>-1</sup> and  $b = 0.02$  ms<sup>-1</sup> where these values apply only for the voltage range investigated in the present study. The reverse rate constant  $l$  is 0.01 ms<sup>-1</sup> in 3-AP and is independent of voltage. The resultant time constant of ~15 ms at +100 mV agrees with the experimental range of values (see Fig. 8). The degree of voltage dependence was not determined in 2-AP, but the rate constants were increased by a factor of 5 at +100 mV to reflect the more rapid apparent interaction of 2-AP with the binding site (Figs. 3 and 6).

It is possible that part of the apparent voltage dependence of block may actually be due to dependence on ionic current through K channels which also increases with increasing depolarization. Our data are not sufficiently detailed to determine such dependence which is currently under investigation.

Simulation of several experiments described previously in Results with this model reveals quantitative agreement of theory and data. The voltage dependence of block observed in potassium current voltage clamp families in *n*-AP can be successfully reproduced as can the slow turn-on of remaining  $I_K$  (Fig. 3).

The progressive removal of *n*-AP block of K channels by repetitive application of step depolarization can be simulated by the model. By virtue of the relatively slow time constant for re-establishment of *n*-AP block after release from the binding site, the large current observed during the second of two pulses separated by a 1-s interval in the model duplicates that seen experimentally (Fig. 6).

In contrast to the substantial recovery from block by repetitive pulsing in 3- and 4-AP, the fractional recovery of  $I_K$  during paired pulses in 2-AP is less (Fig. 6c). In addition, the turn-on kinetics of  $I_K$  in 2-AP is somewhat faster than in 3- and 4-AP and apparently reflects a weaker interaction of 2-AP with the binding site resulting in more rapid release and binding kinetics. This weaker interaction is simulated well by the model (Figs. 3d and 6f), if the rate constants  $k$  and  $l$  are increased by a factor of 5 and the rate for re-establishment of block is increased by a factor of 4.

The reduced apparent potency of *n*-AP under conditions of high external  $K^+$  concentration can be produced by the model without alteration of the AP-binding site parameters. Since at the resting potential ( $\sim 0$  mV) in elevated  $[K]_0$  the majority of K channels will be in the open ( $M + M'$ ) state, the dissociation constant of the release reaction favors nonoccupancy of the binding site of *n*-AP. Thus the steady-state block as assessed by voltage steps  $\sim 0$  mV has essentially already been established for "instantaneous" measurements.

An important implication of our proposed model arises from the location of the release reaction between  $M$  and  $M'$ , namely, that open channels are required for removal of *n*-AP block. Experimental evidence supporting this assumption is difficult to obtain since the clearing of *n*-AP from K channels is quite slow relative to normal channel gating kinetics. We did, however, attempt to simulate our experiments using the same model concept and structure as reaction 5 but placing the release reaction between other intermediate states (i.e.  $L' \rightarrow L$ ,  $W' \rightarrow W$ , or  $V' \rightarrow V$ ). Positioning the release reaction between these nonconducting states failed to simulate experimental data. This suggests that if our conceptual basis of binding and release phenomena between two channel populations with normal gating characteristics is correct, the clearing of *n*-AP molecules from their binding sites upon depolarization occurs predominantly if not exclusively from open K channels.

The ability of the proposed model to duplicate the behavior of  $g_K$  in the presence of aminopyridines is highly satisfactory to this point. In the case of 2-AP, the steady-state block of  $I_K$  is very close to the prediction of the theory. According to the rate constants  $k$  and  $l$  in the kinetic model, the steady-state inhibition of  $I_K$  by 1 mM 2-AP at 100 mV is expected to be 25%. The experimental observation was  $21.6 \pm 2.9\%$  (see Table I). However, an important quantitative disagreement between the experiments and theory occurs in the case of 3- and 4-AP and deserves mention. The steady-state suppression of  $I_K$  in 3-AP as seen in long ( $>50$  ms) depolarizations is approximately 50%, while the model predicts a decrease of 10% at +100 mV when  $k$  and  $l$  are adjusted to match properly the turn-on kinetics and frequency-dependent recovery of  $I_K$ . Three factors which may directly or indirectly affect the measurement of K current in different external K concentration bear on this point.

**POTASSIUM INACTIVATION** A slow inactivation of potassium permeability has been reported for squid axons (Ehrenstein and Gilbert, 1966), and for nodes of Ranvier by Schwarz and Vogel (1971). In the present experiments, the potassium conductance measured at 0 mV in 340 mM  $K^+$  ASW is smaller than that observed in normal  $K^+$  ASW at 0 mV from the holding potential of  $-80$  mV. This result also suggests that inactivation occurs in the potassium-conducting system.

The time constant of removal of aminopyridine block is in the order of 10-20 ms for 3- or 4-AP, therefore a long duration of pulse is required for the K current to reach the steady-state level. For example, at the end of the 70 ms pulse where the steady-state value is measured, an appreciable inactivation of the potassium conducting system may have occurred, which would be reflected in a decrease of K conductance.

**POTASSIUM ACCUMULATION IN A PERIAXONAL SPACE** The accumulation of potassium ions in the periaxonal space after a prolonged step depolarization is well documented in squid axons (Frankenhaeuser and Hodgkin, 1956; Adelman et al., 1973). Such accumulation results in a shift of the thermodynamic equilibrium potential for potassium ions to more positive potentials, a decrease in the driving force for K ion movement at the clamp potential, and a diminished  $I_K$ . Therefore, measurements of  $I_K$  at long times ( $>50$  ms) would be less than apparent steady-state values evaluated at the end of 8-ms pulses, and perhaps closer to the steady-state value in *n*-AP. An instantaneous K current measurement at the end of a long pulse for both control and aminopyridine-treated axons would minimize errors in the determination of  $g_K$ -induced equilibrium potential shifts and potassium inactivation.

**ION-ION INTERACTION IN K CHANNELS** Anomalies in the measurement of  $K^+$  fluxes across metabolically poisoned squid axon membranes have led to the proposal that  $K^+$  ions cross the membrane in a "single file" manner through potassium channels (Hodgkin and Keynes, 1956). Such an arrangement might conceivably give rise to appreciable coulombic interactions between  $K^+$  ions competing for and passing through the channel. If an *n*-AP molecule were located at a site within or near the mouth of a K channel and its affinity for that site were greater than that of a  $K^+$  ion, it could perhaps alter the apparent energy profile of an open and conducting channel as "seen" by  $K^+$  ions. A reduction of the steady-state single channel conductance to  $K^+$  ions might result and would contribute to the experimental reduction in steady-state  $g_K$  in *n*-AP. Such a phenomenon is easily incorporated into the model by reducing the scaling factor  $\bar{g}_K$  of the H-H scheme. Similarly, such an interaction could also affect the binding of *n*-AP with K channels, resulting in a weaker affinity of *n*-AP for its binding site.

*Aminopyridines and TEA Block K Channels Differently*

As mentioned previously, tetraethylammonium ion (TEA) is known to reduce current through K channels in many excitable membranes. Despite this apparent similarity of action to *n*-AP there are a number of important differences between the block of K channels by *n*-AP and TEA (or its  $C_9$  derivative, triethylnonylammonium ion) in squid axons.

(a) Aminopyridines are effective when applied either from the internal or external surface of the membrane, whereas TEA is effective only when applied inside.

(b) Aminopyridines depolarize the squid axon membrane, resulting in repetitive firing and only a slightly prolonged action potential duration. TEA, on the other hand, does not affect the resting membrane potential, but greatly prolongs the duration of action potentials.

(c) Aminopyridine-induced block is relieved by higher depolarizations and increasing pulse duration, while TEA-induced block (especially the  $C_9$  derivative) is enhanced under these conditions. This voltage-independent effect can account for the differences in effect upon the duration of the action potential, as *n*-AP block is decreased during the overshoot phase, while TEA block is increased.

(d) The opening of K channels is required for TEA block to occur (Armstrong, 1966), whereas the opening of K channels favors removal of aminopyridine block.

(e) Aminopyridines reduce both inward- and outward-going K currents, whereas TEA blocks only the outward movement of K<sup>+</sup> ions.

In summary, we have characterized the dynamic interaction of aminopyridines with K channels of the squid axon membrane in terms of a simple kinetic model incorporating the release of *n*-AP molecules from open channels and binding to closed channels. The binding and release scheme derived from our data is sufficient to account for most of our experimental observations without the need to postulate changes in channel gating kinetics. This implies that our model should be independent of the formulation used to describe normal channel gating and as such is not restricted to the linear multistate reaction sequence used in the present simulations, but could use any scheme between closed and open states which satisfactorily describes normal I<sub>K</sub> kinetics.

Frequency-dependent recovery from *n*-AP block has been reported for squid axon (Meves and Pichon, 1975; Yeh et al., 1976*a*; this paper) and frog node of Ranvier (Wagner and Ulbricht, 1975). This phenomenon has not been reported to occur in *Myxicola* axons (Schauf et al., 1976), cockroach axons (Pelhate and Pichon, 1974), and frog skeletal muscle (Gillespie and Hutter, 1975), nor does it occur in lobster giant axons (G. S. Oxford, unpublished observation). This species-dependent effect is intriguing and may provide useful information on the molecular architecture of potassium channels in different excitable membranes.

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