Aminopyridines block an inactivating potassium current having slow recovery kinetics

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ABSTRACT The blocking action of aminopyridines on an inactivating K current (I_{Ki}) in GH_3 pituitary cells was studied before and after altering the macroscopic decay of the current with *N*-bromoacetamide (NBA). The first depolarizing pulse delivered either seconds or minutes after beginning 4-aminopyridine (4AP) application, elicited a current with both a more rapid decay and a reduced peak amplitude. The rapid decay (or time-dependent block) was especially prominent in NBA-treated cells. With continued drug application, subsequent test pulses revealed a stable block of peak current, greater in NBA-treated than control cells. Recovery from block was enhanced by hyperpolarizing holding potentials and by the first depolarizing pulse delivered after prolonged recovery intervals. Unlike aminopyridine block of other K currents, there was no convincing evidence for voltage shifts in activation or inactivation, or for voltage and frequency-dependent unblock. Increasing the open probability of the channels did, however, facilitate the block. Although the behavior of currents in 4AP was suggestive of "open channel block," the block was not produced by 4-aminopyridine methiodide, a positively charged aminopyridine. Moreover, because partial block and recovery occurred without opening the channels we suggest that aminopyridines bind to, or near, this K channel, that this binding is enhanced by opening the channel, and that a conformational change is induced which mimics inactivation. Because recovery from block is enhanced by negative potentials, we suggest that aminopyridine molecules may become "trapped" by inactivation awaiting the slow process of reactivation to escape their binding sites.

INTRODUCTION

Block of delayed rectifier and I_A type potassium currents by 4-aminopyridine (4AP) has been described in a wide variety of cell types ranging from neurons and T lymphocytes to cardiac ventricular cells (e.g., Yeh et al., 1976; Chandy et al., 1984; Josephson et al., 1984). In such cells, aminopyridines have proven useful agents for the separation of K channel subtypes. For example, the K channel which may be responsible for prolonging the cardiac ventricular action potential plateau at short diastolic intervals is a 4AP sensitive transient outward current (Hiraoka and Kawano, 1987). Aminopyridines have also proven useful in augmenting the release of transmitter at nerve terminals (Thesleff, 1980; Bondy and Russell, 1988) and hormones from pituitary cells (Sand et al., 1980; Schofield and Saith, 1981) suggesting that aminopyridine-sensitive K channels may play an important role in the regulation of neurosecretion.

Recently a potassium current I_{Ki}, with complex inactivation gating kinetics was described in GH_3 pituitary cells (May and Oxford, 1986; Wagoner and Oxford, 1987; Oxford and Wagoner, 1989). In whole-cell recording conditions, this current is blocked by either internal or external application of aminopyridines. Moreover, in on-cell or excised membrane patches, minicurrents, representing several activated channels, are also blocked by 4AP.

In previous reports on many different cell types, aminopyridines have been shown to interact with K channels in complex ways resulting in such phenomena as frequency and voltage-dependent unblock. In contrast, we now describe in GH_3 cells a block of I_{Ki} by 4AP which exhibits neither voltage- nor frequency-dependent unblock, but instead reveals a marked time-dependent block. We suggest that the absence of the usual pulse-dependent unblocking features in this channel may reflect more than one pathway to and from the 4AP blocking site(s), as well as the unusually slow process of recovery from inactivation characteristic of these channels (Oxford and Wagoner, 1989).

METHODS

GH_3 cells were obtained from the American Type Culture Collection (Rockville, MD). Culture and recording conditions have been previously described (Oxford and Wagoner, 1989). We used either a List EPC-7 (Medical Systems, Greenvale, NY) or an Axopatch (Axon Instruments Inc., Burlingame, CA) voltage clamp in combination with either a custom 12-bit analog-to-digital converter interfaced with a PDP-11/23 computer or a Scientific Solutions Labmaster A/D board interfaced to an IBM-AT (IBM Instruments Inc., IBM Corp., Danbury, CT). Data

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acquisition and analysis routines were developed under the C-LAB programming environment (INDEC Systems, Sunnyvale, CA). Before digitization, current signals were low-pass filtered at 5–10 kHz (−3 dB, 4-pole Bessel). A −P/4 averaging procedure (Armstrong and Bezanilla, 1977) was used to delete residual linear leakage and capacitive currents not completely corrected by analogue compensation. Approximately 80% of the total series resistance was electronically compensated.

The standard extracellular recording solution contained (mM): 4 $MgCl_2$, 1 EGTA (ethyleneglycol-bis-(beta-aminoethylether)*N*-*N*'-tetraacetic acid) (free Mg^{2+} ~3.7), 5 TEA (tetraethylammonium) Cl, 5 KCl, 135 NaCl, and 300 nM tetrodotoxin. The standard internal solution was (millimolar): 120 K aspartate, 20 KCl, 1 $MgCl_2$, 2 Mg · ATP, 1 cyclic AMP, 10 EGTA (free Mg^{2+} ~1.5), 5 disodium creatine phosphate (CP), 20 U/ml creatine phosphokinase (CPK). All solutions contained 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid) buffer and 10 mM D-glucose with pH and osmolality adjusted to ~7.4 and 300 mosM, respectively. Assuming 3 μM contaminating calcium from salts used to make the solutions, the free $[Ca^{2+}]$ in the external solution was $<2 \times 10^{-10}$ M and in the internal solution was $<2 \times 10^{-11}$ M. The stability constants used in the calculation of free $[Ca^{2+}]$ were from Owen (1976) and Chaberek and Martell (1959). We have previously demonstrated that use of these solutions, along with the voltage protocols described in this paper, adequately eliminates any significant contributions of currents, other than I_{Ki} to the records (Oxford and Wagoner, 1989). Aminopyridines were obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals including *N*-bromoacetamide (NBA) and 4-acetamido-4'-isothiocyanostilbene-2-2'-disulphonic acid (SITS) were obtained from Sigma Chemical Co. (St. Louis, MO). The quaternary 4-aminopyridine methiodide (4-APMI) was prepared according to the methods of Poziomek (1963) and recrystallized from ethanol. NBA and 4AP were prepared daily for each experiment and were applied to the external membrane surface either by continuous bath infusion or via a ∪-tube as previously described (Oxford and Wagoner, 1989). In some experiments 4AP was applied internally using a system of catheters to exchange the pipette solution during an experiment. The design of the exchange apparatus was similar to that described previously (e.g., Cull-Candy et al., 1981).

The terminology associated with ion-channel gating and drug block is, unfortunately, not standardized and multiple meanings for several terms have evolved. We have adopted the following interpretations to associate with certain terms in this paper. *Frequency dependence* refers to the influence of multiple repetitive voltage steps at intervals of 0.5–5 s on the state of aminopyridine block. *Use dependence* is a more general term that refers to the dependence of aminopyridine block on activation of the channel regardless of frequency. *Inactivation* is used to describe the phenomenon of decay of macroscopic K current during a maintained depolarization in the absence of aminopyridines and does not here imply a particular channel state transition in any kinetic scheme for gating.

RESULTS

External application of aminopyridines to GH_3 cells induces two changes in the transient outward current, I_{Ki}. First, aminopyridines produce a time-dependent block which accelerates the decay of the macroscopic current. Second, these compounds cause a more slowly progressing block which reaches equilibrium (stable block) after several minutes and is recorded as a reduction in peak current.

Time-dependent, open channel block of I_{KI} by 4AP

After rapid application (time constant ~100 ms) of 0.05–1.0 mM 4AP via an ∪-tube, voltage steps delivered within 5–15 s elicited currents having an increased rate of decay when compared to control currents. For example, although 1 mM 4AP only slightly reduced peak current after 10 s (Fig. 1 *A*), it noticeably increased the macroscopic decay rate. Similar results were obtained with 3AP (data not shown).

The increase in the rate of decay of I_{Ki} was more pronounced in cells treated with NBA (Wagoner and Oxford, 1987; Matteson and Carmeliet, 1988; Oxford and Wagoner, 1989). For example, after the dramatic slowing of the current decay by 50 μM NBA, a 10-s exposure to 1 mM 4AP reestablished a marked time-dependent decline (Fig. 1 *B*) which closely approximated the normal time course before NBA treatment. The acceleration of decay rate was dose dependent as seen in another NBA-treated cell in Fig. 1 *C*. In this case, a 15-s application of 100 μM 4AP increased the rate of the decay of the current during the voltage step. After washing away the 4AP, the current recovered the slow decay characteristic of NBA-treated cells. A subsequent 15-s exposure of the same cell to 1 mM 4AP yielded a dramatic acceleration of the decay. Computer subtraction of 4AP traces from their corresponding NBA traces yielded time courses of 4AP block

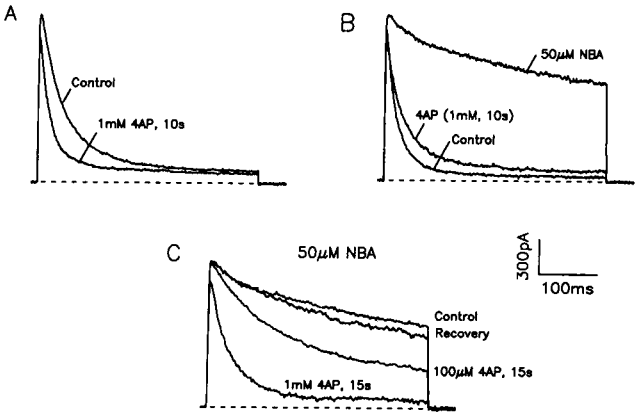

FIGURE 1 Time-dependent block of I_{Ki} by 4AP. (*A*) Decay of I_{Ki} in control cells is accelerated during early exposure to 4AP. Records represent current responses at +40 mV for control conditions (*upper trace*), and after 10 s (*lower trace*) of exposure to 1 mM 4AP. (*B*) Early block of I_{Ki} by 1 mM 4AP in NBA-treated cells mimics the normal decay of current. Traces represent control I_{Ki}, current during application of 50 μM NBA, and current after a 10 s exposure to 1 mM 4AP + 50 μM NBA. (*C*) Current records were obtained at +20 mV after NBA treatment and subsequent 15 s exposures to 100 μM or 1 mM 4AP as indicated for each trace. I_{Ki} was allowed to recover between applications of 4AP. In all panels, holding potential = −60 mV and dashed line represents zero current.

which were fit by single exponentials with time constants of 61.4 and 24.2 ms for 0.1 and 1 mM 4AP, respectively. These observations are reminiscent of the local anesthetic induced "inactivation" of Na currents in pronase-treated axons (e.g., Cahalan, 1978) or the effects of long-chain quaternary ammonium compounds on delayed rectifier K currents (Armstrong, 1971).

A quaternary aminopyridine does not block I_{KI}

Open channel or voltage-dependent block mechanisms are typically associated with the charged state of drug molecules. In particular, quaternary compounds with permanent positive charges have been used to demonstrate the requirement of charge for block. Such compounds have also been useful in determining from which side(s) of the membrane the blocking reaction proceeds (e.g., Yeh, 1982). For example, 4-aminopyridine methiodide (4-APMI) has been shown to block delayed rectifier K channels only when applied to the cytoplasmic side of the channels (Kirsch and Narahashi, 1983). However, when we examined block of I_{Ki} by 4-APMI, we found no evidence of block by this analogue at concentrations as high as 5 mM whether applied externally ($n = 5$) or internally ($n = 4$). This finding suggests that neutral aminopyridine molecules may be the most active form in blocking I_{Ki}. However, the possibility that steric rather than coulombic barriers prohibit block by 4-APMI cannot be excluded.

Closed channel block of I_{KI} by aminopyridines

During 4AP application the first test pulse to assess block revealed not only the increase in rate of decay described above, but also a reduction of peak current. This pattern was observed with either brief (Fig. 2 *A*) or prolonged (Fig. 2 *B*) applications of 4AP. This reduction in peak current was present even when using very negative holding potentials (e.g., −90 mV) which greatly reduced open channel probability. Exponentials were fit to the decay of each trace in Fig. 2 and extrapolated to the beginning of the voltage step to estimate the degree of block before channel opening. A 28% block preceded macroscopic activation during the 30 s of exposure to 300 μM 4AP in A ($V_h = -60$ mV), and a 36% block during the 8-min exposure to 300 μM 4AP in B ($V_h = -70$ mV). Similar analyses on three cells not pretreated with NBA revealed an average "closed channel block" of 42% during exposures to 1 mM 4AP for 5–7.5 min.

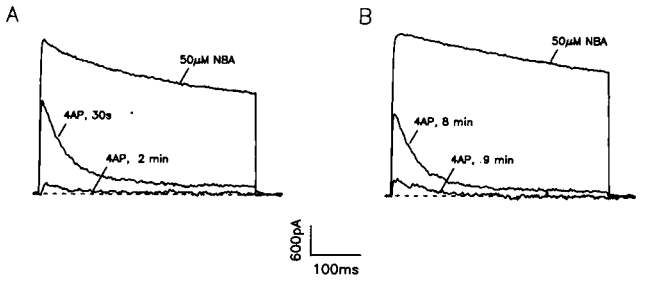

FIGURE 2 4AP block of "closed" channels proceeds slowly, but is accelerated with channel opening. (*A*) Records represent consecutive and exclusive current responses at +40 mV after pretreatment with 50 μM NBA, and after 30 s and 2 min of exposure to 300 μM 4AP + 50 μM NBA as labeled. Holding potential = −60 mV. (*B*) Records from another NBA treated cell in which the first and second test pulses during 300 μM 4AP exposure were applied at 8 and 9 min, respectively. As in *A*, these records were consecutive and exclusive. Holding potential = −70 mV.

Channel opening facilitates block

In spite of our evidence suggesting that 4AP can block closed K channels, channel opening greatly facilitated block. As can be seen in Fig. 2 *B*, after prolonged application of 4AP, substantial I_{Ki} was elicited with the first test pulse at 8 min. However, with the next voltage step, delivered 1 min later, only a weak current was elicited. The block had apparently increased after the activation of channels in the first pulse. A further indication of the enhancement of block by channel opening is the greatly diminished second pulse current (at 2 min) in Fig. 2 *A* as compared to the first pulse current (at 8 min) in Fig. 2 *B*, despite the fourfold longer 4AP exposure in Fig. 2 *B*. Thus, comparison of the degree of block seen during the first voltage step for short versus long duration 4AP exposures indicates that "closed channel" block proceeds quite slowly. In contrast, comparisons between the first and second pulse currents in Fig. 2, *A* or *B*, indicate that "open channel" block proceeds more rapidly.

Stable block of I_{KI} by aminopyridines

Continued exposure to 4AP leads to a progressive decline in peak current resulting in a stable, or steady-state block. This is unlike the case of phasic block of other channels by quaternary ions or local anesthetics where kinetic manifestations of the block persist throughout the drug treatment. Under equilibrium conditions, during continued exposure to 4AP, currents were effectively reduced at all potentials (Fig. 3 *A* and *B*), and could be blocked with either external (Fig. 3 *C*) or internal (Fig. 3 *D*) application. The percentage of peak current blocked by pro-

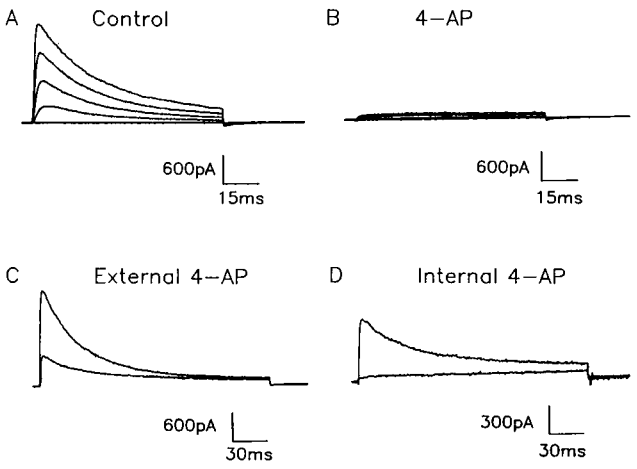

FIGURE 3 External or internal 4-aminopyridine blocks I_{Ki} in GH_3 cells. (*A*) Family of control currents in response to 100 ms voltage steps from −40 to +60 mV in 20 mV increments from a holding potential of −60 mV. Interpulse interval was 35 s. Standard internal and external solutions were used. (*B*) Currents from same cell as shown in *A*, but after bath application of 3.5 mM 4AP. Identical voltage paradigm as in *A*. (*C*) In another cell, 1 mM 4AP (*bath applied*) reduced I_{Ki} 68% after 10 min. Test potential was +40 mV. (*D*) Application of internal 1 mM 4AP to another cell via an exchange pipette (see Methods) dramatically blocked I_{Ki} after 4 min. Pipette exchange time ~3 min. Test potential was +40 mV.

longed application of 4AP is plotted as a function of 4AP concentration in Fig. 4 *A*. 4AP blocked control I_{Ki} in a dose-dependent manner with 50% of the channels blocked at 210 μM (open symbols). 3-Aminopyridine (3AP) produced a similar though less potent block (32% block at 300 μM; data not shown). In cells treated with NBA to slow the decay of the macroscopic current, the steady-state blocking effect of aminopyridines was greatly enhanced. For example, whereas 100 μM 4AP only slightly reduced peak current in a control cell, it blocked over 50% of the peak current in an NBA-treated cell (Fig. 4 *B*). This enhanced effect of 4AP in NBA-treated cells is observable over the entire dose range examined (Fig. 4 *A*, solid symbols).

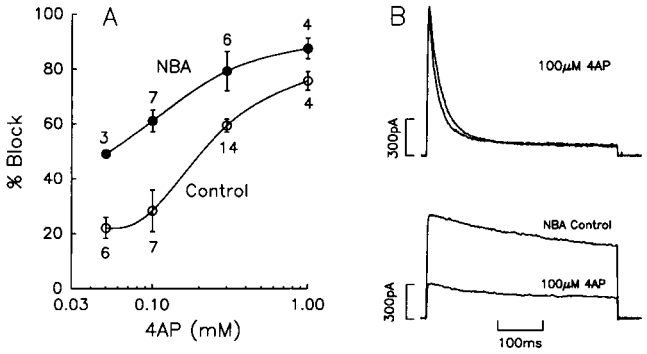

FIGURE 4 Aminopyridine block is enhanced by NBA treatment. (*A*) Dose-response curve for 4AP block of I_{Ki} in control (*open circles*) and NBA-treated (*solid circles*) GH_3 cells. Symbols represent mean ± SD with number of observations indicated for each condition. The lines were drawn through the data points by a spline interpolation routine. (*B*) External 100 μM 4AP produced only a slight change in I_{Ki} at +20 mV under control conditions (*upper traces;* the faster decaying trace was obtained in 4AP). In another GH_3 cell pretreated with 50 μM external NBA, 100 μM 4AP induced a 67% reduction of I_{Ki} (*lower traces*).

Is 4AP block of I_{Ki} voltage dependent?

As mentioned previously, one of the characteristics of aminopyridine block of delayed rectifier channels is voltage dependence. The block becomes weaker for more depolarizing membrane potentials (Yeh et al., 1976). To address this issue for I_{Ki}, two tests of the voltage dependence of block were performed. The experiments were (*a*) to deliver families of voltage steps and look for differential block in various regions of the current-voltage relationship, and (*b*) to vary the holding potential and look for differences in the degree of block at a fixed test potential.

After prolonged application of aminopyridines during which a stable block was achieved, peak current-voltage curves were generated using voltage step families (Fig. 5 *B*). To minimize any possibility of cumulative inactivation during the protocol, the steps were delivered at intervals of greater than 30 s, and to random, rather than consecutive, voltages. Ratio analysis of the resultant

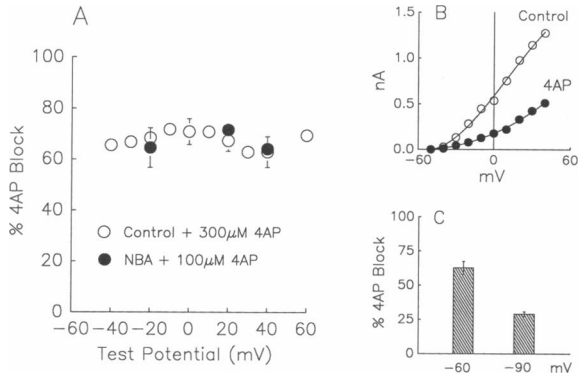

FIGURE 5 Aminopyridine block of I_{Ki} is not dependent on test voltage, but is influenced by holding potential. (*A*) Data points represent percent block (mean ± SD) of peak I_{Ki} as a function of test potential for control cells (*open circles*, 300 μM 4AP, $n = 4$) or cells pretreated with 50 μM NBA (*solid circles*, 100 μM 4AP, $n = 3$). (*B*) Current-voltage relationships for peak I_{Ki} from a control GH_3 cell before (*open circles*) and after (*solid symbols*) exposure to 300 μM 4AP. Lines through the data points are fits of 2nd order polynomials. (*C*) Percent block of peak I_{Ki} for three cells (mean ± SD) exposed to 200 μM 4AP at holding potentials of −60 and −90 mV.

current-voltage curves in the presence and absence of 4AP indicated only little, if any, voltage dependence favoring block relief with increasing depolarization (Fig. 5 *A*). Experiments performed in elevated extracellular K^+ (50–75 mM) to assess block of inward K currents, likewise failed to show significant voltage dependence.

Although test potentials failed to demonstrate the voltage dependence seen for other K channels blocked by aminopyridines, changes in holding potential did affect the percent block by 4AP. When identical concentrations of 4AP were applied twice to the same cells while holding at either −60 or −90 mV (with recovery periods between drug applications), a greater percent block was seen at the more depolarized holding potential (Fig. 5 *C*). Thus, whereas test voltage had little influence on the degree of block, depolarized holding potentials potentiated 4AP block.

Aminopyridines do not alter macroscopic activation, deactivation, or inactivation of unblocked channels

Several compounds which block sodium channels from the intracellular side, have been suggested to gain access to their blocking sites via the aqueous ion pathway, occlude the passage, and prevent closure of the inactivation gate (Yeh and Narahashi, 1977; Armstrong and Croop, 1982). One observation that is characteristic of this scheme is the progressive slowing of deactivation kinetics with increasing depolarizing pulse length. We examined I_{Ki} tail currents at −80 mV (50 mM external K^+) after depolarizing prepulses of varying durations in the presence and absence of 4AP. No differences in either the deactivation time constants nor the inactivation time course (determined from the dependence of peak tail current amplitudes on prepulse duration) were seen. Likewise, no "hooklike" increases in tail currents were observed as seen for many open channel blockers (Yeh and Narahashi, 1977). In addition, we have found no evidence that in the presence of 4AP, activation of unblocked I_{Ki} channels was altered; i.e., there was no change in the rate of rise to peak current or the voltage dependence of peak conductance.

Drug binding can alter ion-channel gating behavior. Likewise, channel gating can alter drug-receptor binding. For example, local anesthetics may shift the voltage dependence of inactivation or slow the recovery from inactivation of sodium channels (Hille, 1977). Such observations have been explained by proposing an enhanced drug binding to inactivated conformations of the channel (Hille, 1984). However, the steady-state I_{Ki} inactivation vs. voltage curve was only marginally shifted by 4AP (Fig. 6 *A*). To minimize contributions from spontaneous shifts in gating kinetics sometimes induced by whole-cell dialysis (Fernandez et al., 1984), control curves were determined at least 15 min after initiating whole-cell dialysis and compared with curves obtained after reaching stable block in 4AP. Control currents were half-inactivated at ~−54 mV, whereas, currents remaining in the presence of 4AP were half-inactivated at ~−61 mV. These differences were not significant.

In the presence of aminopyridines, I_{KI} does not exhibit frequency dependence

Repetitive voltage stimuli have been shown to induce accumulation of sodium-channel block by quaternary derivatives of local anesthetics (Yeh, 1982) and of calcium-channel block by calcium antagonists such as D-600 (Hescheler et al., 1982). In contrast, in the continued presence of the drug, such procedures cause the relief of delayed rectifier K channel block by aminopyridines. This latter finding led to the suggestion that aminopyridines block closed channels, and are released from open channels (Yeh et al., 1976). Subsequent studies have, however, indicated that open delayed rectifier channels are the likely target for aminopyridine block, and that opening the channel permits expression of a voltage-dependent block escape by the charged drug form (Kirsch et al., 1986). Using protocols similar to those of Yeh et al. (1976; i.e., repetitive pulses of 0.5–5 Hz) we investigated dynamic unblock phenomena. Unfortunately, examination of the effects of repetitive voltage steps (at these

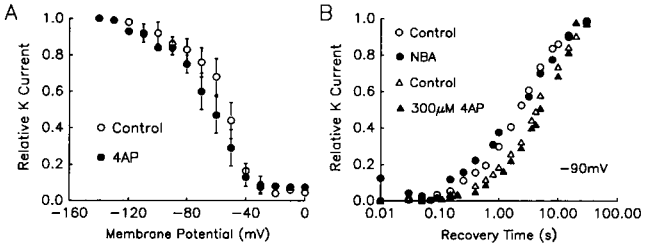

FIGURE 6 Absence of 4AP effects on I_{Ki} inactivation. (*A*) Steady-state inactivation curves for GH_3 cells before (*open circles*) and after (*solid circles*) exposure to 300 μM 4AP. Data represents peak I_{Ki} (mean ± SD, $n = 5$) at +40 mV after a 2 s prepulse to the indicated potential. Control data points were obtained following at least 15 min of dialysis. (*B*) Time course of I_{Ki} reactivation at −90 mV after a depolarizing prepulse to 0 mV for 2 s. Data points from 2 cells (*triangles and circles*) represent peak I_{Ki} normalized to both the control (*maximum*) and inactivated (*minimum*) levels in each case. Open symbols correspond to control data whereas solid symbols correspond to external exposure to either 50 μM NBA (*circles*) or 300 μM 4AP (*triangles*). Note that recovery time is plotted on a logarithmic scale.

frequencies) on drug block of I_{Ki} is complicated by cumulative inactivation which occurs at interpulse intervals <40 s. We nonetheless executed two tests to evaluate frequency-dependent block or block relief. Fig. 7 shows the results of such efforts to reveal 4AP unblock by repeatedly (1 Hz) giving brief (10 ms) depolarizing pulses or by giving depolarizing prepulses of various durations (see Fig. 7 insets for voltage paradigms). In control cells (Fig. 7 *A*), repetitive pulses yielded the expected decreases in current magnitude, indicating that inactivation can occur despite the brevity of pulses. (In all cases in this figure, the largest current is the first in the series of records.) After application of 4AP, repetitive pulsing or depolarizing prepulses either slightly decreased the remaining current amplitude, or had no effect at all. No obvious use dependence was seen.

We reasoned that perhaps our failure to observe block relief with repetitive depolarization (as has been seen for other types of K channels) was due to a "trapping" of 4AP molecules in the channels by the closure of the inactivation gates, or simply due to a balanced expression of two opposing processes, inactivation and block relief. It follows that if inactivation could be removed or slowed, then frequency-dependent phenomena might be unmasked. Therefore, we internally dialyzed either SITS or NBA (Oxford and Wagoner, 1989) to slow macroscopic inactivation and reexamined the effects of repetitive pulse paradigms. Although, as expected, in control cells treated with SITS or NBA (Fig. 7, *B* and *C*), repetitive 10 ms depolarizing pulses did not produce cumulative inactivation, after the application of 4AP, there was still no block relief with depolarizing pulses. There was also no frequency-dependent block.

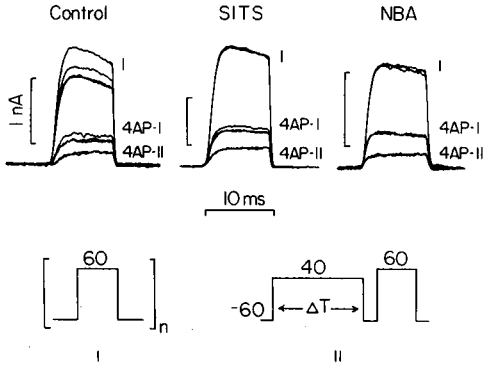

FIGURE 7 Aminopyridine block of I_{Ki} does not exhibit use dependence. Upper row of traces represent current responses to 10-ms voltage steps to +60 mV applied either repetitively (1 Hz, paradigm *I*) or after prepulses of increasing duration (5–65 ms) to +40 mV (paradigm *II*). Groups of superimposed records are labeled according to the paradigm employed and the presence of 1 mM 4AP in the bath. Data shown for three representative cells corresponding to (*left to right*) control conditions, and intracellular application of either 1 mM SITS or 1 mM NBA. Voltage paradigms are diagrammed below current records. For paradigm *I*, current traces are the first 3 or 4 in each case, with subsequent traces (up to 20) showing no change. For paradigm *II*, traces are those at 5, 15, and 65 ms.

To complete our evaluation of the kinetic properties of 4AP block, we delivered single long depolarizing pulses in the presence of 4AP to determine changes in the extent of block. Prolonged depolarization in either control or NBA-treated cells did not produce time-dependent reductions in 4AP block as have been seen for other types of K currents (data not shown).

In the presence of a continual application of NBA, I_{Ki} will eventually inactivate, although much more slowly (Oxford and Wagoner, 1989). Therefore, an alternative hypothesis to explain the absence of voltage- or time-dependent unblock in NBA-treated cells, is that recovery from block is rate-limited by the slow process of recovery from inactivation (reactivation) for the channels underlying I_{Ki}. That is, the abnormally prolonged inactivation, characteristic of these channels, "traps" the aminopyridine molecules at their binding sites. In Fig. 6 *B*, the relative magnitudes of the recovery currents in two cells are plotted as a function of time spent at −90 mV after an inactivating voltage step (0 mV, 2 s). One cell was exposed to NBA to slow inactivation whereas the other was treated with 4AP to block ~50% of the channels. It is apparent that recovery from inactivation remains slow despite the presence of NBA to slow the onset of inactivation, or 4AP to block a large portion of the total current. This observation lends support to the idea that if channels which bind 4AP can still inactivate or assume an inactivation-like state, perhaps frequency- and voltage-dependent behaviors are not observable due to the slow process of recovery from inactivation.

Recovery from aminopyridine block is modulated by voltage and channel gating

After brief applications (seconds) of 4AP which induce increases in the rate of decay of the macroscopic current, complete recovery occurs within seconds and shows no apparent voltage dependence. However, after a stable block of the peak current has been achieved, recovery takes many minutes, is enhanced by very negative holding potentials, and by activating the current with a positive voltage step. In Fig. 8, the percent of peak current at +40 mV is plotted as a function of time in a cell exposed to 200 μM 4AP while holding at −90 or −60 mV. After control periods, 4AP was applied via a ∪-tube until a stable block was achieved. Then the drug was rapidly removed and perifusion continued for 10 min before the first recovery test pulse was given. At a holding potential of −90 mV, more than half of the blocked current recovered

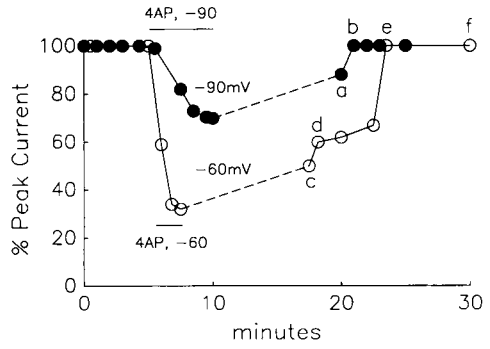

FIGURE 8 Membrane hyperpolarization enhances recovery from 4AP block. A GH_3 cell was consecutively exposed to 200 μM 4AP at holding potentials of −90 mV (*horizontal bar, top*) or −60 mV (*horizontal bar, bottom*) and allowed to fully recover after each exposure. Data points represent the percent peak I_{Ki} at HP = −90 mV (*solid circles*) and −60 mV (*open circles*). In each case no depolarizing voltage steps were delivered during the initial 10 min recovery period in the absence of 4AP (*dashed lines*). Currents elicited by the second test pulses (*b, d*) were significantly larger than those elicited by the first pulses (*a, c*). During the recovery from 4AP at −60 mV, the holding potential was switched from −60 to −90 mV (*e*), enhancing the recovery, and then back to −60 mV for the final point (*f*).

with the first test step (*a*). The second recovery test step (*b*), and all subsequent steps from −90 mV demonstrated complete (100%) recovery. In contrast, when holding at −60 mV, only 26% recovery had occurred when the first recovery test pulse (*c*) was given. Although with the second recovery pulse from −60 mV (*d*), there was a marked increase in total current, the rate of recovery on subsequent pulses remained slow. Upon returning the holding potential to −90 mV (*e*), the current returned rapidly to control levels where it remained despite a return of the holding voltage to −60 mV (*f*). In summary, a negative holding potential combined with a positive voltage step to open channels enhanced recovery from 4AP block.

DISCUSSION

The nature of the interaction between aminopyridines and certain subtypes of K channels is a complicated interplay between membrane potential, channel gating, drug form, and stimulation rate. This interplay results in such phenomena as use-dependent unblock (Yeh et al., 1976) and anomalous depolarization-dependent block restoration (Kirsch et al., 1986). Whereas these observations have guided the design of voltage protocols used in the present study, several previously reported drug block phenomena have not been seen with I_{Ki}. Instead, a transient "open channel block" has been observed that gives way to an equilibrium reduction of current that exhibits neither the voltage nor frequency dependence seen in delayed rectifier channels. Our data suggests that our inability to detect these phenomena may be a reflection of the dominance of the inactivated state which serves to mask behaviors requiring the open state of the channel as a detector. However, aminopyridines do exhibit a form of use-dependent block evidenced by the enhanced time-dependent decay of current during single depolarizations and the pulse-dependent rates of block and recovery.

Comparison of 4AP block of delayed rectifier, I_A and I_{KI} channels

The characteristics of 4AP block of K channels reflect the relative rates of channel gating and 4AP binding and unbinding. Differences in the gating rates of delayed rectifier, I_A, and I_{Ki} channels appear in part to underlie differences in the expression of voltage or frequency dependence. For example, in the delayed rectifier potassium channel, the relief from aminopyridine block with depolarization is slower than normal activation gating. Likewise, the reestablishment of block with hyperpolarization is slower than deactivation kinetics. Therefore, features such as use-dependent recovery with repetitive depolarization are seen because channel gating is at equilibrium during much of the dynamics of the drug-channel interaction. Because inactivation is not a prominent gating feature of these channels (Chabala, 1984), it does not significantly influence the block.

Unlike the dissimilar kinetics of gating and 4AP block of the delayed rectifier potassium current, the voltage- and time dependence of gating and aminopyridine block are more comparable for I_A. Thus, features of block dynamics which are easily seen in delayed rectifier channels (e.g., frequency-dependent unblock), are much less exaggerated in I_A channels (Thompson, 1982). This difference appears to be related to the presence of inactivation which "competes" with drug molecules for block of the passage: channels are either blocked or inactivated. Because recovery from inactivation is rapid in I_A, occurring within 1 s near the resting potential, repetitive pulsing elicits currents that can reflect drug-channel interactions rather than cumulative inactivation. The mirror image voltage relationship for inactivation and aminopyridine block led Thompson (1982) to propose that modulation of block by inactivation either reflects prevention of drug access to the receptor site by a closed inactivation gate, or dependence of the formation of a 4AP receptor on the open state of the inactivation gate. The concept that inactivated and drug-blocked channel states are exclusive was further supported by the observation that I_A recordings during repetitive pulses in 4AP exhibited "crossover," such that greater block of peak

current was associated with less inactivation at longer times during a pulse. A similar argument has been used to explain the interactions between monovalent cations and the inactivation gates of sodium channels (Oxford and Yeh, 1985).

In I_{Ki} of GH_3 cells, as in I_A, the onset of aminopyridine block and of macroscopic inactivation gating are very similar. Therefore, one might expect to see classic aminopyridine block or unblock phenomena. However, even in cells treated with NBA to slow inactivation, these dynamic features are still not readily apparent. This is in part due to the extremely slow recovery from inactivation (reactivation) which prevents an unambiguous assessment of block kinetics at stimulation rates comparable to those used for other channels.

There are at least two possibilities for the failure to see the classic dynamic features of block and unblock by aminopyridines. First, just as depolarization opens channels and draws unblocked channels into an inactivated conformation from which recovery is slow, blocked channels may also proceed to a slow "recovery" state in which aminopyridines become effectively unable to dissociate. That is, the rate constant for 4AP dissociation from the channel is extremely slow at all voltages. Alternatively, blocked channels may still be able to inactivate and thus cannot conduct regardless of the state of block. This latter possibility is supported by (*a*) the identity of reactivation rates in control, NBA, and 4AP treated cells, (*b*) the absence of "hooks" or progressive slowing in I_{Ki} tail currents, and (*c*) the absence of I_{Ki} crossover during depolarizing pulses when 4AP records are compared to controls.

The binding site(s) for aminopyridines in I_{KI} channels

In I_{Ki}, 4AP produces, on a millisecond time scale, both transient open-channel block (reflected by faster decay of the current), and a steady-state block (reflected by a decrease in peak I_{Ki}), which are concentration dependent (Figs. 1 and 4). The steady-state block can apparently proceed from the closed state, although very slowly (Fig. 2). This dual nature of 4AP block suggests that either two binding sites exist in or near the channel, or two pathways are available for the drug to reach a common binding site. Perhaps there are both hydrophilic and hydrophobic pathways for the aminopyridine drug/receptor interaction, one via the open K channel, and one through the lipid bilayer or channel wall. Our data do not allow discrimination between these possibilities. The apparent increase in sensitivity to 4AP block in NBA-treated cells likely reflects the increase in open channel probability induced by NBA (Oxford and Wagoner, 1989), and, therefore, the time for drug binding to open channels. The dual nature of block is also apparent in the investigation of recovery from block. Only a portion of the total peak current recovers unless depolarizing pulses are given at frequencies which do not produce cumulative inactivation.

The effect of holding potentials on the percent of steady-state block and block recovery reflect the importance of both channel opening and the slow process of recovery from inactivation to the kinetics of 4AP block. Channel-opening probability is likely higher at −60 mV than −90 mV and, thus, even without activation of currents detectable at the macroscopic level, more channels should be available for "open channel block" mechanisms. This is consistent with the observation that the degree of block is greater for more depolarizing holding potentials. A similar conclusion was reached by Kirsch et al. (1986) for the effects of holding potential on aminopyridine block of delayed rectifier channels. Additionally, at −60 mV, almost half of the available channels move into the inactivated state, and the rate of reactivation is relatively slow. If 4AP molecules are "trapped" by the inactivated state of the channel, this should also contribute to the enhanced steady-state block at this holding potential. Channels are less likely to escape inactivation at −60 mV than at −90 mV and, thus, clear themselves of 4AP. Accordingly, the rate of recovery from 4AP block is slower at −60 mV than at −90 mV. The effect of membrane hyperpolarization (which removes inactivation) to enhance recovery from block is similar to that seen for the recovery from local anesthetic block (Hille, 1977).

In summary, we have described aminopyridine block of a K current having slow inactivation recovery kinetics. This block is unique in its simplicity, lacking some of the voltage and frequency-dependent characteristics found for other K channel subtypes. However, channel gating phenomena do influence the rate and degree of the block. 4AP block of I_{Ki} apparently proceeds and recovers from both closed and open channel states and is enhanced by compounds such as NBA which slow the macroscopic current decay.

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