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4-Aminopyridine Binding and Slow Inactivation Are Mutually Exclusive in Rat Kv1.1 and *Shaker* Potassium Channels

NEIL A. CASTLE, SANDRA R. FADOUS, DIOMEDES E. LOGOTHETIS, and GING KUO WANG

Department of Anesthesia Research Laboratories, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115 (N.A.C., S.R.F., G.K.W.), and Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, New York 10029–6574 (D.E.L.)

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SUMMARY

In the present study we have used two-electrode voltage-clamping of *Xenopus* oocytes expressing either Kv1.1 or *Shaker* B (ShB) Δ6–46 K^+ channels to examine the effects of 4-aminopyridine (4-AP) on the process of slow inactivation. Neither of these channels exhibits fast inactivation. Channel activation was required for block by 4-AP in both channel types. In the absence of drug, inactivation of Kv1.1 and ShB Δ6–46 channels at 0 mV was biexponential [τ_{fast} = 7.8 ± 0.3 sec, τ_{slow} = 33.9 ± 0.9 sec, and $A_{slow}/(A_{fast} + A_{slow})$ = 0.79 ± 0.02 (n = 10) for Kv1.1 and τ_{fast} = 3.5 ± 0.4 sec, τ_{slow} = 13.1 ± 1.8 sec, and $A_{slow}/(A_{fast} + A_{slow})$ = 0.35 ± 0.06 (n = 3) for ShB Δ6–46]. In the presence of 4-AP, the rates of inactivation of Kv1.1 and ShB Δ6–46 were markedly slowed, resulting in a crossover phenomenon where, in the presence of drug, the outward current was smaller than control at the beginning of the depolarizing pulse but crossed over during the pulse to become larger than the control. The most obvious change induced by 0.2 mM 4-AP was a 2-fold slowing of the slow phase of inactivation [τ_{fast} = 3.9 ± 1.1 sec, τ_{slow} = 67.1 ± 3.6 sec, and $A_{slow}/(A_{fast} + A_{slow})$ = 0.85 ± 0.04 (n = 4) for Kv1.1 and τ_{fast} = 3.5 ± 0.4 sec, τ_{slow} = 23.7 ± 2.6 sec, and $A_{slow}/(A_{fast} + A_{slow})$ = 0.75 ± 0.02 (n = 3) for ShB Δ6–46, in the presence of 0.2 mM 4-AP]. In addition, there was a significant increase in the contribution of the slower phase of inactivation of ShB Δ6–46 channels in the presence of 4-AP. The slowed inactivation in the presence of 4-AP was accompanied by removal of 4-AP block. These results are consistent with the processes of 4-AP block and slow inactivation of Kv1.1 and ShB Δ6–46 channels being mutually exclusive.

The time course of inactivation varies among different voltage-dependent K^+ channel types. For example, delayed rectifier K^+ currents typically do not inactivate during maintained depolarizations sometimes lasting for several seconds, whereas transiently activated K^+ channels often inactivate within tens of milliseconds. Studies of potassium channels cloned from the *Shaker* locus of *Drosophila* have provided evidence for the existence of at least two distinct mechanisms of inactivation (1–3). Rapid inactivation that occurs with time constants ranging between 5 and 100 msec is most frequently associated with a process termed N-type inactivation. This form of inactivation is believed to involve the amino-terminal region of the K^+ channel subunits acting as a tethered "ball" (or particle) that swings over to occlude the permeation pathway (1). Removal of N-type inactivation of *Shaker* K^+ channels by selective deletion of the subunit amino terminus or by exposure of the intracellular face of the channel to proteases reveals a second, slower, form of inactivation. This latter type of inactivation has been termed C-type inactivation (3).

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K^+ channel inactivation can be reversibly modified by a variety of pharmacological agents. For example, intracellular application of TEA not only reduces the peak current amplitude of *Shaker* K^+ channels but also slows the process of fast inactivation, apparently by competing with the amino-terminal inactivation particle for the same or overlapping intracellular binding sites (2). In contrast to intracellular TEA, extracellularly applied TEA has no effect on the time course of fast inactivation of ShB channels; however, it does slow the kinetics of the slower C-type inactivation (2). A similar effect of extracellularly applied TEA on the inactivation of rat Kv1.3 K^+ channels expressed in oocytes and n-type K^+ currents in T lymphocytes has been observed (4, 5). Additional studies showing that site-directed mutations of the pore region and S6 transmembrane domain of ShB channels (3, 6, 7) and changes in extracellular potassium concentrations (6, 8, 9) also modify slow inactivation of *Shaker* channels have resulted in speculation that an extracellularly facing region at the carboxyl-terminal end of the channel subunit is involved in C-type inactivation.

Like TEA, 4-AP is widely used as a selective blocker of voltage-dependent K^+ channels. Block of K^+ channels by 4-AP

ABBREVIATIONS: TEA, tetraethylammonium; ShB, *Shaker* B; 4-AP, 4-aminopyridine; TPeA, tetrapentylammonium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

involves a complicated interplay between membrane potential, channel gating, and the form of drug present (10–20). Furthermore, the apparent mechanism of block appears to differ depending on the channel type being examined. Studies on native K^+ channels have reported both open channel block (12, 21) and resting closed channel block (10, 15, 16) by 4-AP. However, all studies examining 4-AP block of cloned K^+ channels indicate that channel activation is required for block (17–20, 22, 23).

In common with the blocking action of intracellularly applied TEA, the processes of 4-AP block and fast inactivation of voltage-dependent K^+ channels have been reported to be mutually exclusive (13, 15, 16, 20, 23, 24). Because 4-AP appears to access its binding site from the intracellular face of K^+ channels (14, 18, 25), it has been argued that, like TEA, 4-AP and the tethered ball responsible for N-type inactivation share common or overlapping binding sites (20, 23). In contrast to its actions on fast inactivation, there is at present little information on the effects of 4-AP on the slower C-type inactivation. Therefore, in this study we have examined the effect of 4-AP on slow inactivation of the cloned rat delayed rectifier K^+ channel Kv1.1 and the ShB K^+ channel deletion mutant ShB Δ6–46 (1). We chose to examine the ShB Δ6–46 K^+ channel mutant because it lacks the amino-terminal domain that is responsible for N-type inactivation but still exhibits the slower C-type inactivation (3). Kv1.1 was chosen because of its high sensitivity to block by 4-AP and its extremely slow rate of inactivation, which occurs over a period of several minutes (21) and may be due C-type inactivation. The results presented in this study show that the processes of 4-AP block and slow inactivation are mutually exclusive in both Kv1.1 and ShB Δ6–46 K^+ channels expressed in *Xenopus* oocytes.

Materials and Methods

Kv1.1 cDNA was subcloned into the *Eco*RI site of the pGEM expression vector (21, 26). ShB Δ6–46 clone (1) was kindly provided by Dr. Richard Aldrich (Stanford University). The cDNAs were linearized and transcribed as reported previously (17). Under anesthesia, ovarian lobes were surgically removed. Oocytes were subjected to digestion for 20 min in a nominally Ca^{2+}-free medium containing 2 mg/ml collagenase. The oocytes were then defolliculated manually. Defolliculated oocytes were stored overnight at 17°. Viable oocytes were then injected with 50 nl of mRNA for the channel to be examined, via a glass micropipette (diameter, 15–25 μm). Injected oocytes were stored at 17° for up to 5 days in modified Barths's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 2.5 mM sodium pyruvate]. For recordings, oocytes were transferred to a chamber that could be perfused continuously. The standard recording solution contained 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.3. Currents were recorded with a conventional two-microelectrode voltage-clamp amplifier (model OC-725A; Warner Instrument Corp., Hamden, CT). Data acquisition and analysis were controlled by pCLAMP software interfaced to the amplifier via a 125-kHz Labmaster board (Axon Instruments, Foster City, CA).

Results

Slow inactivation of Kv1.1 and ShB Δ6–46 K^+ channels. Both Kv1.1 and ShB Δ6–46 K^+ channels were activated by depolarization to membrane potentials more positive than −40 mV (Fig. 1). For pulse durations of <1 sec, little or no inactivation of either channel type was evident (Fig. 1A). However, when the depolarization was maintained for periods of >2 sec a slowly developing reduction in current amplitude could be observed. For both channel types, inactivation developed in a biexponential manner (for time constants, see Table 1). Inactivation of Kv1.1 currents was approximately 3-fold slower than that of ShB Δ6–46 currents. Futhermore, whereas the slower phase of inactivation contributed approximately 80% of the total inactivation of Kv1.1 currents, its contribution for ShB Δ6–46 channels was only 35%. The rates of slow inactivation of both channel types showed no measurable voltage dependence, which is consistent with previous reports (3, 21). The time constants for slow inactivation of Kv1.1 currents described here are similar to the values reported for inactivation of Kv1.1 currents in Sol-8 cells stably transfected with Kv1.1 cDNA (21). However, the rate of inactivation of ShB Δ6–46 currents reported here is slower than the rate previously reported for this current by Hoshi *et al.* (3) but is similar to the value reported by Boland *et al.* (27).

The voltage dependence of "steady state" activation and

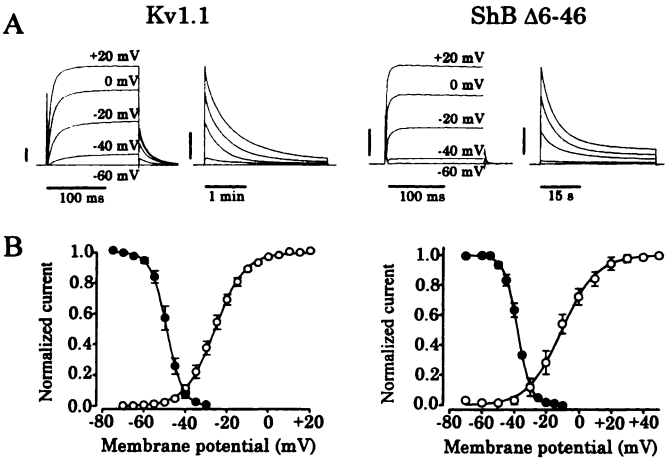


Fig. 1. Slow inactivation of Kv1.1 and ShB Δ6–46 K^+ channels. A, Superimposed families of current traces elicited by increasingly more depolarized voltage steps, shown on two different time scales, i.e., 150-msec and 3-min pulses for Kv1.1 channels and 180-msec and 45-sec pulses for ShB Δ6–46 channels. Vertical bars equal 2 μA. B, Steady state activation (○) and inactivation (●) curves for Kv1.1 and ShB Δ6–46 channels. Activation curves were determined from the amplitudes of tail currents elicited upon repolarization to −60 mV after 150-msec conditioning voltage steps to potentials ranging from −70 mV to +50 mV. Inactivation curves were determined from the peak amplitudes of currents elicited by a 240-msec test pulses to 0 mV after 3-min (Kv1.1) or 1-min (ShB Δ6–46) conditioning voltage steps to potentials ranging from −70 to −10 mV. Data were normalized to the maximal current value estimated from a fit of the Boltzmann equation to the data. For the inactivation data, the noninactivating current component was subtracted before the normalization procedure. The curves are least squares fits of the Boltzmann equation to the data. The midpoint potentials and slopes are given in the text.

TABLE 1

Time course of slow inactivation in the presence and absence of 0.2 mM 4-AP

	τ_{fast}	τ_{slow}	$A_{slow}/(A_{fast}+A_{slow})$	n
	sec	sec		
Kv1.1				
Control	7.8 ± 0.3	33.9 ± 0.9	0.79 ± 0.02	10
4-AP	3.9 ± 1.1[a]	67.1 ± 3.6[a]	0.85 ± 0.04	4
ShB Δ6-46				
Control	3.5 ± 0.4	13.1 ± 1.8	0.35 ± 0.06	3
4-AP	3.5 ± 0.4	23.7 ± 2.6[a]	0.75 ± 0.02[a]	3

[a] Significantly different from control ($p < 0.05$).

inactivation for both channel types is shown in Fig. 1B. Steady state activation was determined by measuring the peak tail current amplitude at −60 mV after either 50-msec (ShB Δ6–46) or 100-msec (Kv1.1) voltage steps to various potentials. Steady state inactivation was assessed by applying either a 3-min (Kv1.1) or 45-sec (ShB Δ6–46) conditioning voltage step to various membrane potentials, immediately followed by a 400-msec test pulse to 0 mV. The midpoint potentials and slopes of the steady state activation curves were −25 ± 0.5 mV and 7.4 ± 0.2 mV (n = 7) for Kv1.1 and −9.3 ± 1.5 mV and 9.0 ± 1.1 mV (n = 7) for ShB Δ6–46, respectively. The midpoint potentials and slopes of the steady state inactivation curves were −49.2 ± 0.5 mV and 3.9 ± 0.3 mV (n = 5) for Kv1.1 and −37.8 ± 0.3 mV and 4.0 ± 0.2 mV (n = 3) for ShB Δ6–46, respectively.

The rates of recovery from slow inactivation of Kv1.1 and ShB Δ6–46 channels are shown in Fig. 2. Recovery was assessed by initially applying either a 3-min (Kv1.1) or 1-min (ShB Δ6–46) conditioning voltage step to 0 mV, followed, after a variable delay at −80 mV, by a 400-msec test pulse to 0 mV. The cycle times for application of the pulse protocols were 10 min for Kv1.1 and 1 min for ShB Δ6–46. The time course for recovery from inactivation for both channel types was best described by a two-exponential function. The time constants for Kv1.1 were τ_{fast} = 6.2 ± 1.1 sec, A_{fast} = 0.58 ± 0.05, τ_{slow} = 104.0 ± 15.7 sec, and A_{slow} = 0.42 ± 0.05 (n = 5), whereas those for ShB Δ6–46 were τ_{fast} = 2.1 ± 0.6 sec, A_{fast} = 0.57 ± 0.16; τ_{slow} = 20.0 ± 15.0 sec, and A_{slow} = 0.43 ± 0.11 (n = 3).

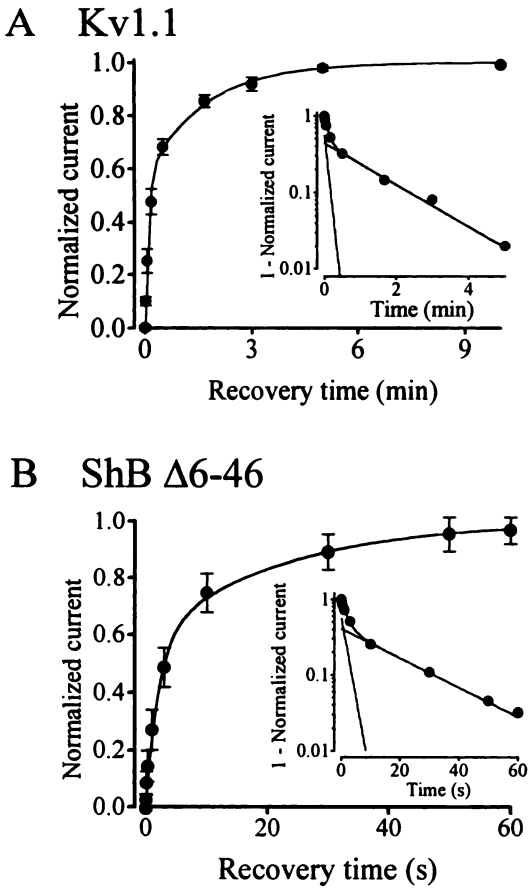


Fig. 2. Rate of recovery from slow inactivation. A, Time course of Kv1.1 channel recovery from inactivation was assessed by application of a 3-min conditioning voltage step to 0 mV, followed, after a variable rest period at −80 mV, by a 200-msec test pulse to 0 mV. The data points are means ± standard errors from five cells. The curve is a least squares fit of a biexponential function to the data, using the following parameters: τ_{fast} = 6.2 ± 1.1 sec, A_{fast} = 0.58 ± 0.05, τ_{slow} = 104.0 ± 15.7 sec, and A_{slow} = 0.42 ± 0.05. *Inset*, semilogarithmic plot of the data. B, Time course of ShB Δ6–46 channel recovery from inactivation was assessed as for Kv1.1 currents, with the exception that the conditioning voltage step had a duration of 1 min. The data points are means ± standard errors from three cells. The data were best described by a biexponential function with the following parameters: τ_{fast} = 2.1 ± 0.6 sec, A_{fast} = 0.57 ± 0.16, τ_{slow} = 20.0 ± 15.0 sec, and A_{slow} = 0.43 ± 0.11. *Inset*, semilogarithmic plot of the data.

Block of Kv1.1 and ShB Δ6–46 K+ channels by 4-AP. A previous study of Kv1.1 K+ channels expressed in Sol-8 cells showed that block of this channel type by 4-AP depends on channel activation (17). This has been confirmed for 4-AP block of Kv1.1 channels expressed in *Xenopus* oocytes in the present study (Fig. 3). A similar mechanism of 4-AP block appears to underlie inhibition of ShB Δ6–46 K+ channels (Fig. 3). Fig. 3A shows the development of 4-AP (0.5 mM) block of Kv1.1 and ShB Δ6–46 currents, monitored by application of 240-msec depolarizing voltage steps to 0 mV every 60 sec for Kv1.1 or 30 sec for ShB Δ6–46. After an initial delay of approximately 90 sec, which was likely attributable to the dead space of the perfusion system and the membrane barriers of *Xenopus* oocytes, current amplitude declined with successive pulses until steady state inhibition was attained after an exposure time of 12 min for Kv1.1 channels and 8 min for ShB Δ6–46 channels. For both Kv1.1 and ShB Δ6–46 currents, inhibition was associated with an increase in the apparent rate of inactivation. This phenomenon became more evident when the oocytes were preexposed to 4-AP before application of a voltage step to assess the magnitude of block (Fig. 3B). Fig. 3B shows current traces elicited by the following pulse protocol. The membrane potential was initially held at −80 mV (to populate the closed channel state). 4-AP (0.5 mM) was then applied externally for 40 min (Kv1.1) or 15 min (ShB Δ6–46)

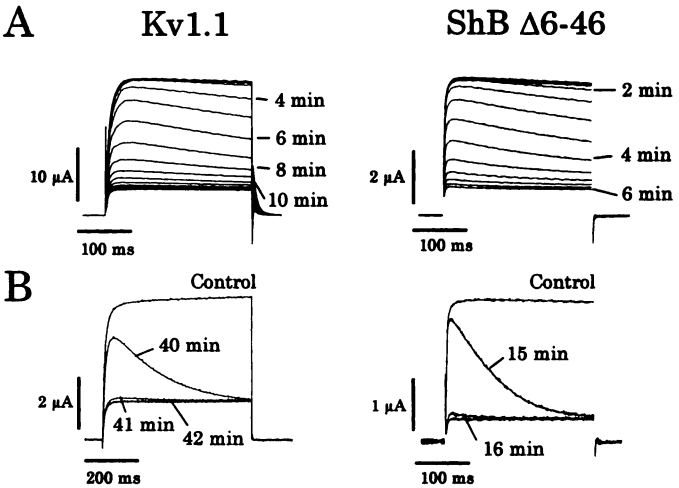


Fig. 3. Requirement of channel activation for 4-AP block of Kv1.1 and ShB Δ6–46 channels. A, Development of block by 0.5 mM 4-AP, monitored by voltage steps from −80 mV to 0 mV applied every 30 sec (ShB Δ6–46) or 60 sec (Kv1.1). B, Development of block after exposure to 0.5 mM 4-AP in the absence of channel opening. Current traces were elicited by a pulse protocol similar to that described in A. The first current trace was generated after 40-min (Kv1.1) or 15-min (ShB Δ6–46) exposure to 4-AP at −80 mV. The second and third current traces were evoked 30 sec and 60 sec (ShB Δ6–46) or 1 min and 2 min (Kv1.1), respectively, after the first trace.

at this potential, in the absence of activating voltage steps. Based on the results shown in Fig. 3A, a 15-min exposure to 0.5 mM 4-AP should be sufficient for the drug concentration to reach steady state at or near its site of interaction for both channel types. After the quiescent exposure period, depolarizing voltage steps to +40 mV were applied every 60 sec (Kv1.1) or 30 sec (ShB Δ6–46) in the continued presence of 4-AP, to assess the degree of inhibition. The current traces elicited by the first depolarizing voltage step after the preexposure to 4-AP exhibited a small initial reduction in peak amplitude, followed by a marked time-dependent decline in current amplitude to a level that approximated the steady state inhibition seen in Fig. 3A. The second and third depolarizing voltage steps, applied 1 and 2 min (Kv1.1) or 30 sec and 60 sec (ShB Δ6–46), respectively, after the first pulse, elicited nondecaying currents with amplitudes similar to current levels observed at the end of the first voltage step. These results suggest that before the first depolarizing voltage step little or no block of the channel occurred. However, upon channel activation the 4-AP bound with an exponential time course until steady state was reached. No further block occurred with subsequent voltage steps. We have previously shown that, upon closure of Kv1.1 channels, 4-AP becomes "trapped," being unable to unbind until the channel reopens (17). Thus, both binding and unbinding of 4-AP occur with the channel in the activated conformation. In the present study we observed a similar closed channel trapping of 4-AP with ShB Δ6–46 channels (data not shown).

Effect of 4-AP on slow inactivation of Kv1.1 and ShB Δ6–46 K^+ channels. Slow inactivation of Kv1.1 and ShB Δ6–46 K^+ channels in the absence and presence of 0.2 mM 4-AP is shown in Fig. 4. In addition to reducing the peak amplitude, 4-AP produced an apparent slowing of the rate of inactivation of both current types. The slowing of inactivation by 4-AP produced a "crossover" phenomenon in which the current amplitude in the presence of blocker was smaller than control at the beginning of the depolarizing pulse but crossed over to become larger than control by the end of the pulse (Fig. 4A). The time course of inactivation in the presence and absence of 4-AP is shown Fig. 4B. Like the control currents, inactivation in the presence of 4-AP was best described by a two-exponential function. The time constants for inactivation in the absence and presence of 0.2 mM 4-AP are given in Table 1. The most noticeable effect of 4-AP on both channel types was an approximately 2-fold increase in the time constant (τ_{slow}) for the slower phase of inactivation. However, in addition there appeared to be channel-specific effects of 4-AP. For example, whereas the total contribution of the slower component of inactivation of Kv1.1 channels remained essentially unchanged in the presence of 4-AP (control, 0.79; 4-AP, 0.85), the relative contribution of this component to inactivation of ShB Δ6–46 channels more than doubled in the presence of the blocker (control, 0.35; 4-AP, 0.75). Although the time constant of the faster phase of inactivation of Kv1.1 channels was approximately 2-fold lower in the presence of 4-AP, the significance of this is uncertain, especially because this component contributed only 20% of the total inactivation. No 4-AP-induced change in τ_{fast} for inactivation of ShB Δ6–46 channels was observed.

Mutual exclusivity of 4-AP binding and slow inactivation. The phenomenon of current crossover in the presence of 4-AP has been previously reported in studies of 4-AP block of transiently activated K^+ currents in neurons, cardiac muscle, and melanotrophs (13, 15, 16, 24). Those studies concluded that the crossover phenomenon results from inactivation being prevented when 4-AP is bound to its receptor on the channel. Although the concept is not implicit in those results, one can extrapolate from the aforementioned conclusion and postulate that inactivation prevents 4-AP binding. If such a phenomenon occurred in Kv1.1 and ShB Δ6–46 channels, it might be expected that the process of slow inactivation would eventually result in displacement of 4-AP from its binding site. To examine whether such a phenomenon occurred, the pulse protocol shown in Fig. 5 was used. Thirty seconds after application of a short test pulse to 0 mV to assess current amplitude in the presence and absence of 4-AP, a second, longer, pulse to 0 mV was applied to produce inactivation of the current. The membrane potential was then repolarized to −80 mV for 5 min (Kv1.1) or 1 min (ShB Δ6–46), to allow the channels to recover from inactivation. A second short pulse to 0 mV was then applied to assess the degree of block. Fig. 3B shows that for exposure periods of >15 min little or no binding of 4-AP to Kv1.1 or ShB Δ6–46 channels occurred at −80 mV. Due to the closed channel trapping of 4-AP, it can be further assumed that minimal dissociation of bound 4-AP occurred at −80 mV (see Discussion for qualifications of this assumption). Consequently, throughout the rest period at −80 mV, during which recovery from inactivation occurred, the level of 4-AP block of Kv1.1 and ShB Δ6–46 channels that existed at the end of the inactivating pulse should have been maintained. Thus, if any dissociation of 4-AP occurred during the inactivating pulse, it should be manifested as a larger amplitude outward current with any subsequent pulse to 0 mV. As can be seen in Fig. 5, currents elicited after the inactivating pulse did indeed initially

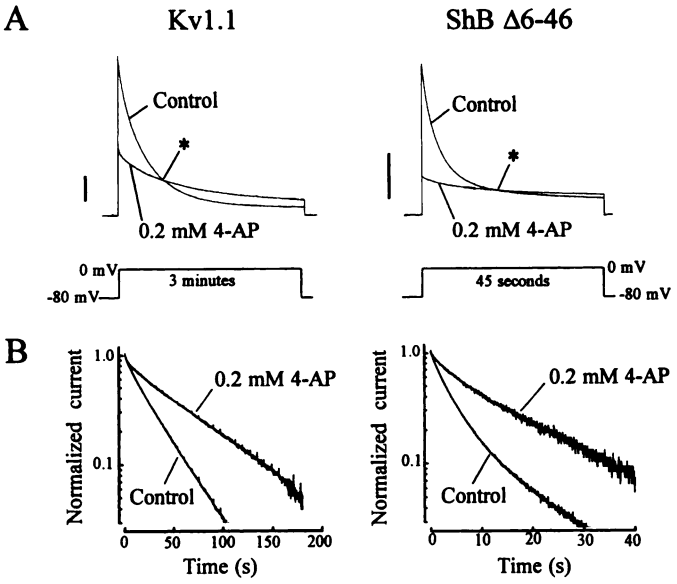


Fig. 4. Slowing of the rate of inactivation of Kv1.1 and ShB Δ6–46 channels in the presence of 4-AP. A, Current traces elicited in the absence and presence of 0.2 mM 4-AP in response to prolonged voltage steps from −80 mV to 0 mV. The amplitudes of the current traces elicited in the presence of 4-AP are smaller than control at the beginning of the pulse but crossover to become larger than control by the end of the pulse. *, Crossover point. Vertical bars equal 2 μA. B, Semilogarithmic plot of the inactivating phases of the current traces shown in A. The current traces elicited in the presence and absence of 4-AP have been normalized to the maximal current amplitude determined from a fit of a biexponential function to the inactivating component of the current. For time constants. see Table 1.

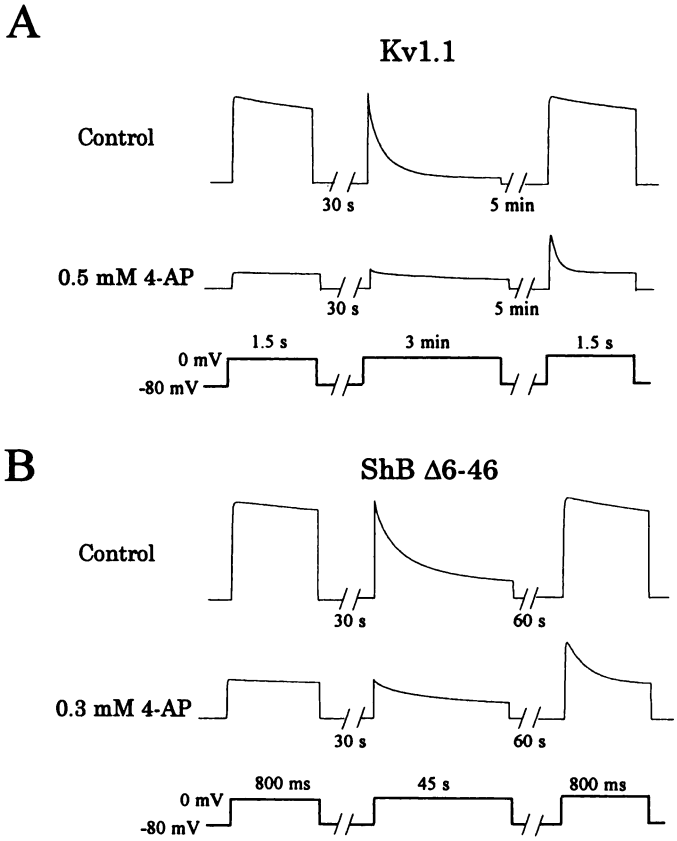


Fig. 5. Displacement of 4-AP from its blocking site by inactivation of Kv1.1 and ShB Δ6–46 channels. A, Twenty seconds after application of a 1.5-sec test pulse to 0 mV to assess Kv1.1 current amplitude in the presence and absence of 0.5 mM 4-AP, a second, longer (3 min), pulse to 0 mV was applied to produce inactivation of the current. The membrane potential was then repolarized to −80 mV for 5 min to allow the channels to recover from inactivation. A second, 1.5-sec, pulse to 0 mV was then applied to assess the degree of block. B, A protocol similar to that used in A was performed with ShB Δ6–46 currents in the presence and absence of 0.3 mM 4-AP. The test pulse and inactivating voltage steps to 0 mV had durations of 800 msec and 45 sec, respectively.

exhibit a larger amplitude. However, the current subsequently decayed in an exponential manner to an amplitude similar to that seen before the inactivating pulse.

If the larger amplitude current elicited after the inactivating pulse did indeed reflect the dissociation of 4-AP during the process of slow inactivation, then one might expect the time courses for development of the transient outward current component and slow inactivation in the presence of 4-AP to be similar. We used a modification of the pulse protocol used in Fig. 5 to test this hypothesis. The duration of the inactivating pulse was varied and the peak amplitude of the inactivating component of the subsequent test pulse current was determined. The amplitudes of these current components were well fit by single-exponential functions, with time constants of 88 sec for Kv1.1 and 12 sec for ShB Δ6–46 currents. Fig. 6 shows the normalized time course for the reduction in test pulse current amplitude superimposed on current traces elicited in the same oocyte during a 3-min (Kv1.1) or 45-sec (ShB Δ6–46) inactivating pulse to 0 mV. The time courses for the two processes are clearly similar.

Discussion

In the present study we have shown that, in addition to reducing current amplitude, 4-AP produces an apparent slowing

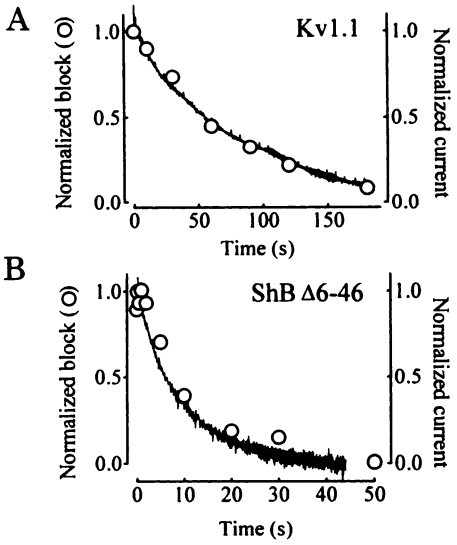


Fig. 6. Similar time courses for slow inactivation and removal of 4-AP block in Kv1.1 (A) and ShB Δ6–46 (B) channels. Using a variation of the pulse protocol used in Fig. 5, the duration of the conditioning inactivating pulse to 0 mV was varied and the peak amplitude of the inactivating component elicited, after a 5-min (Kv1.1) or 1-min (ShB Δ6–46) rest at −80 mV, by a test pulse to 0 mV was determined. The time-dependent changes in the amplitudes of the inactivating current components were well fit by single-exponential functions with time constants of 88 sec for Kv1.1 and 12 sec for ShB Δ6–46 currents. The normalized time course for the reduction in test pulse current amplitude has been superimposed on current traces elicited in the same oocyte during a 3-min (Kv1.1) or 45-sec (ShB Δ6–46) inactivating pulse to 0 mV.

of the rate of inactivation of Kv1.1 and ShB Δ6–46 K^+ channels. The slowing of inactivation is associated with a crossover of current waveforms, where the current amplitude in the presence of 4-AP is smaller than control at the beginning of an activating pulse but crosses over to become larger by the end of the pulse. Furthermore, inactivation results in the removal of block by 4-AP.

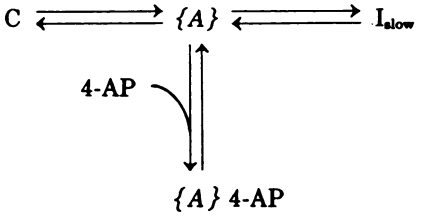
The 4-AP-induced slowing of inactivation and associated crossover phenomenon observed in the present study are reminiscent of 4-AP block of rapidly inactivating K^+ currents (often termed I_{TO} or I_A) in cardiac myocytes, lymphocytes, and some types of neurons (14–16, 24). It has been argued that the 4-AP-induced slowing of inactivation of these rapidly inactivating K^+ currents in neurons and cardiac myocytes is a consequence of the processes of channel inactivation and 4-AP binding being mutually exclusive (15, 16, 24). Thus, in 4-AP-blocked channels inactivation can occur only after 4-AP unbinding, the frequency and duration of which are determined by the dissociation and association rate constants for 4-AP. However, because the process of inactivation can be considered to be essentially irreversible, compared with the kinetics of 4-AP binding, once inactivation occurs the channel is "locked" into a nonconducting state that can no longer bind 4-AP. Thus, 4-AP just delays the inevitable transition to the inactivated state. The 4-AP-induced slowing of inactivation is reminiscent of a similar phenomenon that occurs during inhibition of cloned *Shaker* K^+ channels by intracellularly applied TEA (2). It has been argued that, when bound, TEA prevents the amino-terminal peptide ball from interacting with its receptor within or near the cytoplasmic vestibule of the permeation pathway, most likely as a result of steric hindrance. A similar mechanism could account for 4-AP-induced slowing of inactivation in rapidly

inactivating K^+ channels. Evidence for mutual exclusivity between the processes of 4-AP binding and N-type fast inactivation in cloned *Shaker* K^+ channels has been provided by the observation that the apparent potency of 4-AP increases in ShB channel mutants that exhibit progressively slower rates of inactivation (20, 22). Indeed, in the present study the half-maximal inhibitory concentration for block of the slowly inactivating amino-terminal deletion mutant ShB Δ6–46 (0.1 mM) was found to be approximately 50-fold lower than the value previously reported for 4-AP block of the rapidly inactivating "wild-type" ShB channels (28). It has also been shown that the magnitude of 4-AP inhibition of mouse Kv1.1 channels is reduced by a factor of 3 after intracellular application of a 20-amino acid peptide corresponding to the amino-terminal domain of the ShB K^+ channel (23).

Although mutual exclusion may account for the 4-AP-induced slowing of Kv1.1 and ShB Δ6–46 channel inactivation, as well as the inactivation-induced "knock-off" of 4-AP from its binding site observed in the present study, it is likely to be distinct from the perceived concept of 4-AP and an inactivating particle competing for the same or overlapping binding sites, as might be the case for a channel exhibiting N-type inactivation. Although at present a conceptualized view of the conformational changes underlying slow (C-type) inactivation of ShB Δ6–46 and Kv1.1 K^+ channels is at best rudimentary, it is generally believed that this type of inactivation involves amino acid residues near the external mouth of the pore (3, 7, 27) and results from a concerted conformational change of the four constituent channel subunits (29). However, as stated above, the blocking site for 4-AP appears to be within or near the intracellular mouth of the pore (14, 18, 25). The apparent disparity in the channel domains involved in 4-AP block and slow inactivation raises the question of how the two processes interact with each other. Based on whole-cell and single-channel current measurements, several studies have concluded that block and unblock of cloned K^+ channels by 4-AP occur while the channel is in an activated or open conformation (17–20, 23). Thus, the 4-AP-bound activated conformation of the channel may be unable to undergo the transition to the inactivated conformation. If this were the case, then such an action would contrast with the reported absence of any change in the kinetics of slow inactivation of ShB Δ6–46 after intracellular application of the open channel-blocking agent TEA (2). Although it could be argued that the differing actions of 4-AP and TEA result from different binding sites, evidence to the contrary has recently been provided by the observation that 4-AP and the TEA homolog TPeA produce a mutually exclusive block of Kv3.1 K^+ channels (30). Furthermore, both 4-AP and intracellularly applied TEA have been reported to mutually exclude the inactivation particle responsible for N-type inactivation from its binding site, which is believed to be within the intracellular vestibule of the permeation pathway (31). However, it is worth noting that, unlike either of the two blocking agents, N-type inactivation appears to enhance the rate of C-type inactivation of ShB channels (3).

How can the apparently mutually exclusive interactions of 4-AP, internally applied TEA, and N-type inactivation be reconciled with the differing effects of these treatments on C-type inactivation? The answer to this question may lie in the state-dependent interaction of 4-AP with K^+ channels. The channel states to which 4-AP binds appear to depend on the K^+ channel type being examined. Although 4-AP block of the transient outward K^+ current in ventricular myocytes (15, 16) and melanotrophs (13) has been reported to occur at hyperpolarized membrane potentials, where the channels are mostly in a "resting" (closed) state, studies with cloned voltage-dependent K^+ channels have consistently shown that channel activation is important for induction of block by 4-AP (17–20, 23). Although several studies of cloned K^+ channels have suggested a role for the open conformation in block by 4-AP (20, 23), a recent study of the gating characteristics of ShB Δ6–46 K^+ channels proposed that 4-AP binds not to the open conformation but to a closed state that immediately precedes the transition to channel opening (19). The binding of 4-AP stabilizes this closed conformation, preventing the quaternary rearrangement that is associated with the opening of the conduction pathway. The prevention of channel opening by 4-AP could explain the apparent anomalies with respect to the mutual interactions of 4-AP, internally applied TEA, and N-type inactivation, as well as the respective abilities of these treatments to modify C-type inactivation. For example, both N- and C-type inactivation are coupled to channel activation (3). By locking the channel into a closed state, 4-AP may prevent the conformational rearrangement (e.g., channel opening) that favors these processes. However, during periods in which 4-AP is dissociated from its binding site, the transition from the closed conformation to the open conformation and ultimately to the inactivated conformation can occur. Once the channel is in the inactivated state, transitions of the channel to the 4-AP-favored closed state are rare and consequently the ability of 4-AP to bind to the channel is reduced. The involvement of distinct channel states could also explain the apparent mutual exclusivity of block by 4-AP and the open channel-blocking TEA homolog TPeA (30). When the channel is blocked by TPeA, the channel is locked in the open state (the channel cannot close until the blocker dissociates) (32, 33). The lack of availability of the closed conformation precludes the binding of 4-AP. Similarly, when 4-AP is bound, channel opening is prevented, reducing TPeA binding. It can be noted that selective interaction of 4-AP with a closed conformation of K^+ channels could also explain the inability of elevated extracellular K^+ concentrations to modify 4-AP block of Kv1.1 channels (17), as would be expected if the binding site is within the permeation pathway (32).

In conclusion, the mutual exclusivity between 4-AP block and slow inactivation of Kv1.1 and ShB Δ6–46 channels can be summarized in the following kinetic scheme,



where C is the resting closed state, I_{slow} is the slow inactivated state, and {A} represents the sets of states to which 4-AP can bind and from which slow inactivation can occur. These states are likely to include the open state and one or more of the nonconducting states through which the channel passes during activation.

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Send reprint requests to: Neil A. Castle, Department of Anesthesia Research Laboratories, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.