Modulation of 4-AP Block of a Mammalian A-Type K Channel Clone by Channel Gating and Membrane Voltage

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ABSTRACT We examined the state-, voltage-, and time dependences of interaction between 4-AP and a mammalian A-type K channel clone (rKv1.4) expressed in *Xenopus* oocytes using whole-cell and single-channel recordings. 4-AP blocked rKv1.4 from the cytoplasmic side of the membrane. The development of block required channel opening. Block was potentiated by removing the fast inactivation gate of the channel (deletion mutant termed "Del A"). A short-pulse train that activated rKv1.4 without inactivation induced more block by 4-AP than a long pulse that activated and then inactivated the channel. These observations suggest that both activation and inactivation gates limit the binding of 4-AP to the channel. Unblock of 4-AP also occurred during channel opening, because unblock required depolarization and was accelerated by more frequent or longer depolarization pulses (use-dependent unblock). Analysis of the concentration dependence of rate of block development indicated that 4-AP blocked rKv1.4 with slow kinetics (at −20 mV, binding and unbinding rate constants were 3.2 mM^{-1} s^{-1} and 4.3 s^{-1}). This was consistent with single-channel recordings: 4-AP induced little or no changes in the fast kinetics of opening and closing within bursts, but shortened the mean burst duration and, more importantly, reduced the probability of channel opening by depolarization. Depolarization might decrease the affinity of 4-AP binding site in the open channel, because stronger depolarization reduced the degree of steady-state block by 4-AP. Furthermore, after 4-AP block had been established at a depolarized holding voltage, further depolarization induced a time-dependent unblock. Our data suggest that 4-AP binds to and unbinds from open rKv1.4 channels with slow kinetics, with the binding site accessibility controlled by the channel gating apparatus and binding site affinity modulated by membrane voltage.

INTRODUCTION

4-Aminopyridine (4-AP) blocks many K channels with different state-, voltage-, and time-dependences. The actions of 4-AP on K channels can be divided into three categories: (1) "Resting-state block" (such as 4-AP block of transient outward current (I_A) of ventricular myocytes from ferret (Campbell et al., 1993) and rat (Castle and Slawsky, 1993) and rat melanotrophs (Kehl, 1990), and delayed rectifier current (I_{KDR}) in squid axons (Yeh et al., 1976)). For these channels, 4-AP block occurs at negative membrane voltages when channels are mostly in their resting-state. 4-AP dissociates from channels upon membrane depolarization when channels open. (2) "Open-state block" (such as 4-AP block of I_A in GH_3 cells (Wagoner and Oxford, 1990), delayed rectifier current (I_K) in B-lymphocytes (Choquet and Korn, 1992) and sciatic nerves of *Xenopus laevis* (Arhem and Johansson, 1989), and ATP-regulated K current ($I_{K(ATP)}$) in skeletal muscles (Davies et al., 1991)). For these channels, 4-AP block requires channel opening. (3) "Mixed state-dependent block" (such as 4-AP block of I_A in molluscan central neurons (Thompson, 1982) and in dog ventricular myocardium (Simurda et al., 1989)). For these channels, 4-AP block occurs both at negative membrane voltages (when channels are mostly in the resting-state) and initially after channel opening, with the blocking rate in the open-state faster than that in the resting-state. Furthermore, unblock occurs during prolonged depolarization.

There are further quantitative or even qualitative differences in 4-AP actions on channels within each of the above categories. For example, in the "resting-state block" category, hyperpolarization slows the development of 4-AP block of I_A in ferret ventricular myocytes (Campbell et al., 1993) and I_{KDR} in squid axon (Kirsch et al., 1986), but accelerates 4-AP block of I_A in rat ventricular myocytes (Castle and Slawsky, 1993). These were accounted for by differences in 4-AP binding affinity among various resting-states of these channels. In the "open-state block" category, 4-AP blocks $I_{k(ATP)}$ with fast kinetics (at 0 mV, binding and unbinding rate constants are 17×10^3 mM^{-1} s^{-1} and 61×10^3 s^{-1}), whereas it blocks I_A in GH_3 cells and I_K in B-lymphocytes with much slower kinetics (binding and unbinding rate constants are three orders of magnitude slower than those for $I_{k(ATP)}$) (Davies et al., 1991; Wagoner and Oxford, 1990; Choquet and Korn, 1992). Moreover, 4-AP block of I_{KATP} is enhanced by membrane depolarization, whereas 4-AP block of the latter two channels is apparently voltage-independent, suggesting differences in 4-AP binding sites in various channels relative to the membrane electrical field. Still within the "open-state block" category, for I_{KDR} in sciatic nerve repolarization leads to 4-AP dissociation from the channel before the activation gate can close (Arhem and Johansson, 1989). However, for I_K in B-lymphocytes, repolarization causes the activation gate to close when the channel is still bound with 4-AP, thus trapping 4-AP inside the channel (Choquet and Korn, 1992). Dissociation occurs when the channel opens again.

In view of the diversity in 4-AP's actions on various K channels, it might be a useful "model" agent with which to

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explore molecular mechanisms underlying the diversity of drug-channel interactions. In this regard, studies using K channel clones may provide an important tool. The state-dependence of 4-AP actions has been studied for several K channel clones (Hice et al., 1992; Castle et al., 1993; Kirsch et al., 1993; McCormack et al., 1994; Kirsch and Drewe, 1993). Here we examined the state-, voltage- and time dependences of 4-AP actions on a mammalian A-type K channel (rKv1.4, formerly known as RHK1) (Tseng-Crank et al., 1990). We compared the responses to 4-AP of wild-type rKv1.4 with those of a deletion mutant in which the fast inactivation process had been disrupted (Del A (Tseng-Crank et al., 1993)). The kinetics of interaction between 4-AP and Del A were characterized at both whole-cell and single-channel levels. Our data suggest that 4-AP actions on rKv1.4 belong to the "open-state block" category. However, there are similarities between 4-AP actions on rKv1.4 and those of the other two categories. A preliminary report has been published in an abstract form (Yao and Tseng, 1993).

MATERIALS AND METHODS

cRNA in vitro transcription and oocyte expression

cDNA sequences of the wild-type (WT) rKv1.4 (Tseng-Crank et al., 1990) and its deletion mutant (Del A) (Tseng-Crank et al., 1993) in pBluescript (Stratagene, San Diego, CA) were used to prepare DNA templates for in vitro transcription. The plasmid DNA was linearized by *Hin*dIII, and transcription reaction was performed using a commercial kit (Riboprobe Gemini system, Promega, Madison, WI) in the presence of T7 RNA polymerase and $m^7G(5')_{ppp}(5')G$ (New England Biolab, Beverly, MA). The quality of cRNA product of each transcription reaction was checked by denaturing agarose gel electrophoresis. cRNA was dissolved in RNase-free water for oocyte microinjection.

The oocytes of *Xenopus laevis* were isolated by partial ovariectomy. Follicular cell layers were removed mechanically alter mild digestion with collagenase (type B, Boehringer Mannheim, Indianapolis, IN). Four to six hours after isolation, oocytes were microinjected with cRNA solutions using a Drummond digital microdispenser. The volume injected was 30–50 nl per oocyte. The oocytes were incubated at 16°C in an ND 96 solution (mM: NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, Na-pyruvate 2.5, pH 7.5 with NaOH), supplemented with penicillin (50 U/ml), streptomycin (50 ug/ml), and gentamycin (10 ug/ml). The oocytes were studied 2–6 days after injection.

Electrophysiological experiments

For whole-cell voltage clamp experiments, the oocytes were placed in a tissue chamber and superfused at room temperature (23–25°C) with ND 96 at a rate of approximately 3 ml/min. Membrane currents were studied using the conventional two-microelectrode voltage clamp technique with an AxoClamp-2A amplifier (Axon Instruments, Foster City, CA). The voltage-recording microelectrode had a fine tip (tip resistance 1–2 MΩ, filled with 3 M KCl). In the majority of experiments reported here, an "agarose-cushion pipette" was used to pass currents (Schreibmayer et al., 1993). The large tip (5–10 μm, 0.2–0.5 MΩ) allowed a high current-passing capability and, thus, fast and adequate voltage control. These pipettes were filled with 3 M KCl. The tips were filled with 1% agarose in 3 M KCl to a height of 0.5–1 mm to prevent leakage of KCl into the cells. During current recordings, the bath solution was changed to a nominally Ca-free ND96 solution ($CaCl_2$ substituted by $MgCl_2$) to minimize interference from endogenous Ca-activated Cl currents (Bolton et al., 1989).

For single-channel recordings, the vitelline membrane of oocytes was manually removed with fine forceps under a dissecting microscope after the oocyte had been shrunk by a hypertonic solution (mM: K-aspartate 200, KCl 20, $MgCl_2$ 1, EGTA 10, HEPES 10, pH 7.4 with KOH) (Methfessel et al., 1986). During recordings, the oocyte was bathed in a high $[K]_o$ solution (mM: KCl 140, $MgCl_2$ 2, EGTA 11, HEPES 10, pH 7.5 with KOH). This solution brought the membrane voltage to approximately 0 mV so that the patch membrane potential could be controlled by the pipette voltage. The pipette was coated with Sylgard (Dow Corning) to about 200–300 μm from the tip and filled with normal ND 96 solution (tip resistance 2–15 MΩ). Single channel currents were recorded in the cell-attached patch configuration (Hamill et al., 1981) using an AxoPatch-1C amplifier (Axon Instruments).

4-Aminopyridine (4-AP) was added from a 0.1 M stock to the bath solution to reach a final concentration of 10 μM to 1 mM. At higher concentrations, 4-AP powder was added directly to the bath solution. The pH of all 4-AP-containing solutions was adjusted to 7.5 with HCl. In some experiments, 4-AP (20 mM in water, pH adjusted to 7.5 with Tris-HCl) was injected into oocytes via a third intracellular electrode. The injecting device was essentially the same as that used for cRNA injection, except that fine-tipped electrodes (tip bevelled, diameter <3 μm) were used to minimize cellular injury.

The voltage clamp protocols are described in figure legends or corresponding text. Voltage clamp protocol generation and data acquisition were controlled by an IBM/AT-compatible computer with Clampex of pClamp via a 12-bit D/A and A/D converter (TL-1 DMA Interface, Axon Instruments). Currents were low-pass filtered with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA) at 2 kHz, digitized on-line and stored on diskettes for off-line analysis. The sampling interval for whole-cell currents ranged from 0.1 to 2.5 ms, depending on the purpose of the voltage clamp protocol and the rate of decay of current under study. The sampling interval for single channel currents was 0.1 ms.

Data analysis

Data analysis was mainly carried out using the pClamp suite of programs. For whole-cell current analysis, Clampan was used to perform leak-subtraction and amplitude measurements. Linear capacitive and leak-currents were subtracted using templates created by averaging 10–20 current traces induced by pulses from −80 to −60 or −70 mV (subthreshold depolarization). Clampfit was used to fit the decay time courses of currents. PeakFit (Jandel Scientific, Corte Madera, CA), was used to fit the concentration-response relationships.

For single-channel analysis, the data were processed by a digital filter included in Fetchan (final filter frequency = 1 kHz). The number of active channels in a patch was estimated by the maximal number of channels opened simultaneously when the patch membrane potential was stepped from a negative holding voltage (−100 to −120 mV) to a strongly depolarized voltage (+40 to +60 mV). Most of the patches contained multiple active channels (2–6). To avoid interference in the measurements of single channel current amplitude and kinetics due to overlapping openings, current traces that contained overlapping events were excluded from analysis. Capacitive transients were largely eliminated electronically using the capacitance compensation circuitry of the AxoPatch-1C amplifier. The uncompensated capacitive transients and leak currents were digitally subtracted using templates created by averaging traces without channel openings (null traces).

Single-channel current amplitudes were determined by fitting amplitude histograms with a Gaussian function using pStat of pClamp. The single-channel data were then idealized using a 50% amplitude criterion to detect opening and closing transitions (Colquhoun and Sigworth, 1983). Open time distributions were fit with a single exponential function to estimate the mean open times. Distributions of closed times shorter than 20 ms were fit with a double exponential function, and the time constants of the fast component were designated as the mean closed times within bursts. Bursts of single channel activities were defined by a "criterion duration": any closed interval longer than the criterion duration was assigned as termination of a burst. Criterion duration was set at 5 times the mean closed time within bursts of each patch (Magleby and Pallotta, 1983; Tseng-Crank et al., 1993). The mean burst duration and mean number of openings per burst were algebral means of these parameters.

When applicable, data are presented as means and SD. Statistical significance was tested using the paired or unpaired *t*-test.

RESULTS

State dependence of 4-AP block of rKv1.4

Effects of 4-AP on wild-type rKv1.4 (WT)

Our first attempt to identify the state dependence of 4-AP block of rKv1.4 was to test whether it required membrane depolarization. rKv1.4 was activated by 500 ms depolarization pulses from V_h −80 mV to V_t −20 mV once every 2 min. After the current stabilized under the control conditions, the bath solution was switched to a 4-AP (0.5 mM)-containing one. It took 20–30 s to totally change the bath solution, as measured by monitoring membrane potential changes upon switching the superfusate containing varying $[K]_o$. The interval between the last pulse before 4-AP application and the first in 4-AP (~2 min) allowed 4-AP to approach an equilibrium concentration around its binding site. The negative V_h (−80 mV) during this period maintained the channels mostly in the resting state. If 4-AP binding occurred in the resting state, block should occur before depolarization and manifest as a reduction in the peak amplitude of current induced by the first pulse in 4-AP. On the other hand, if 4-AP binding required membrane depolarization, the peak amplitude of current induced by the first pulse after 4-AP application should be similar to control, but declined during subsequent pulses. Fig. 1 *A* illustrates the effects of 4-AP on wild-type rKv1.4 (WT) studied using such a protocol. The peak amplitude of current induced by the first pulse in 4-AP was the same as control. The current decayed at a faster rate than control. When the current traces were fit with a single exponential function, the time constant of decay was 62.2 ms for the control trace and 53.1 ms for the trace induced by the first pulse in 4-AP. The peak current amplitude started to decrease during the second pulse in 4-AP, and continued to decrease for the next two pulses. A steady-state block by 4-AP was reached at the fourth pulse after 4-AP application (peak current amplitude reduced by 28%). These observations indicate that block of rKv1.4 by 4-AP could not occur when the channel was in the resting state but required membrane depolarization.

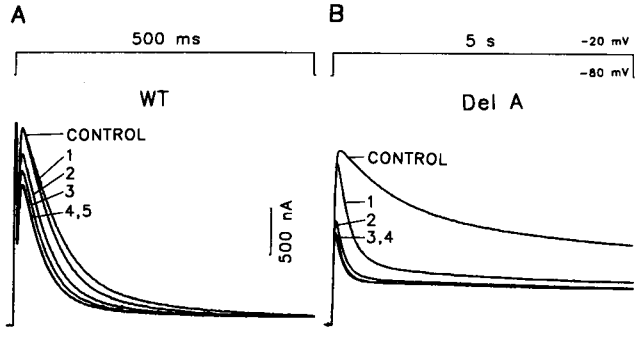


FIGURE 1 Effects of 4-AP on the wild-type rKv1.4 (WT, panel *A*) and its deletion mutant (Del A, panel *B*). Voltage clamp protocols are shown above the current traces. Currents were induced by depolarization pulses from a holding voltage (V_h) −80 mV to a test voltage (V_t) −20 mV once every 2 min. The test pulse durations were 500 ms for WT and 5 s for Del A. Shown are superimposed current traces recorded before (control) and after adding 4-AP (0.5 mM). The numbers (1 to 4 or 5) denote the sequence of pulses applied after adding 4-AP.

Effects of 4-AP on a deletion mutant of rKv1.4 (Del A)

4-AP-induced acceleration of rKv1.4 decay during depolarization could be due to either "open state" block (if 4-AP unblock was slower than channel inactivation), or "inactivated state" block (if 4-AP stabilized the channel in the inactivated state). To differentiate between these two possibilities, we tested the effects of 4-AP on a deletion mutant of rKv1.4, Del A. In this mutant channel, 23 amino acids had been deleted from the "ball" domain in the NH_2-terminus of rKv1.4. The fast inactivation process was disrupted (Tseng-Crank et al., 1993). However, the voltage dependence of activation or single channel conductance was not altered (Tseng and Tseng-Crank, 1992; Tseng-Crank et al., 1993). During depolarization, Del A spends much more time in the open state than WT rKv1.4 because of a prolonged open time within bursts, prolonged burst durations, and increased reopening probability during depolarization (Tseng-Crank et al., 1993). If 4-AP blocked rKv1.4 by binding to the inactivated state of the channel, its effects should be reduced or abolished when tested on Del A. On the other hand, if 4-AP blocked rKv1.4 by binding to the open state of the channel, prolonging the time the channel spent in the open state in Del A should enhance the potency of 4-AP.

Fig. 1 *B* illustrates the effects of 4-AP on Del A. 4-AP concentration was the same as that used in the experiments on the WT channel (0.5 mM). The voltage clamp protocol was also similar except that the duration of depolarization pulses was prolonged to 5 s. The peak current amplitude of Del A induced by the first pulse in 4-AP was only slightly reduced relative to control. However, the decay of the current was markedly accelerated. The time to decay to 50% of peak amplitude was reduced from 2450 to 280 ms. There was a pronounced reduction in the peak current amplitude during the second pulse in 4-AP. 4-AP block reached a steady state by the third pulse, with a 66% reduction in the peak amplitude.

Similar observations were obtained in four experiments on WT and six experiments on Del A. The peak current amplitude during the first pulse after 4-AP application was comparable to control (WT) or only slightly reduced (Del A). For WT, the time constant of current decay during the first pulse in 4-AP was shortened from 52 ± 18 to 45 ± 17 ms. It required 5.6 ± 0.9 pulses for 4-AP block to reach a steady state when the peak current amplitude was reduced by 29 ± 4%. For Del A, the time to decay to 50% of peak during the first pulse in 4-AP was shortened from 2600 ± 420 to 320 ± 150 ms. 4-AP block of Del A took 3.7 ± 0.5 pulses to reach a steady state. At this stage, the peak current amplitude was reduced by 62 ± 14%. Therefore, 4-AP was more potent in blocking Del A than WT rKv1.4, and the rate to reach the steady state 4-AP block (in number of pulses) appeared to be faster in Del A than in WT.

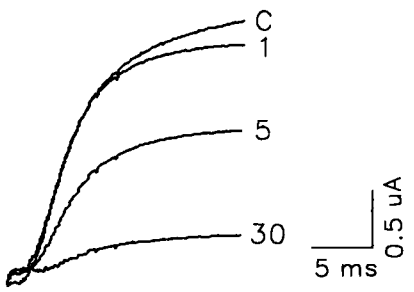


FIGURE 2 Effects of 4-AP on the activation phase of Del A. Shown are superimposed leak-subtracted current traces recorded before and after adding 4-AP (0.5 mM). Currents were activated by 20-ms pulses from V_h −80 mV to V_t −20 mV once every 10 s. The current amplitude was stable under the control conditions (trace marked by "C"). The numbers adjacent to the traces denote the sequence of pulses applied in the presence of 4-AP. Between control and the first pulse in 4-AP, the cell was exposed to 4-AP for 5 min during which the membrane voltage was held at −80 mV.

Although the data presented so far suggest that 4-AP blocked rKv1.4 in the open state, we still need to account for the small reduction in peak current amplitude of Del A during the first pulse in 4-AP (Fig. 1 *B*). This could mean that 4-AP could block Del A in the resting state, although at a much slower rate and to a much lesser degree than the block in the open state (Thompson, 1982; Simurda et al., 1989; Wagoner and Oxford, 1990). The rising phase of Del A before and after 4-AP application (0.5 mM) is shown at an expanded time scale in Fig. 2. The current trace during the first pulse in 4-AP was superimposable on the control trace for the initial 8 ms. It then deviated from the control trace so that the "peak" current amplitude in 4-AP was lower than control. As the degree of channel block increased during the following pulses, the current amplitudes were smaller than control even in the very beginning of the pulses. These observations indicate that the small reduction in the peak amplitude of Del A during the first pulse in 4-AP could be attributed to open state block occurring during the activation phase of Del A. This phenomenon was not apparent in WT at the same 4-AP concentration (0.5 mM), probably because the fast inactivation process truncated the activation phase of the WT current. However, at higher 4-AP concentrations (>1 mM) the rate of block development was accelerated so that the peak amplitude of WT during the first pulse in 4-AP started to show an obvious reduction.

Comparison of 4-AP actions between WT and Del A

The difference in 4-AP's blocking potency between WT and Del A was further confirmed by constructing the concentration-response relationships of 4-AP block. The voltage clamp protocols are described in Fig. 3, *A* and *B*, and summarized data are shown in Fig. 3 *C* (3–7 observations for each of the 4-AP concentrations). For each channel, the relationship between 4-AP concentration and the amount of remaining current at the steady-state block was fit with the following equation:

$$\text{Remaining fraction} = 1/(1 + [\text{4-AP}]/K_d),$$

where K_d is the apparent dissociation constant of 4-AP. This equation assumed that there was a 1:1 binding stoichiometry between 4-AP and the channels. This was confirmed in another series of experiments on Del A and will be discussed later (Fig. 7). The estimated K_d was 1.2 mM for WT and 0.35 mM for Del A.

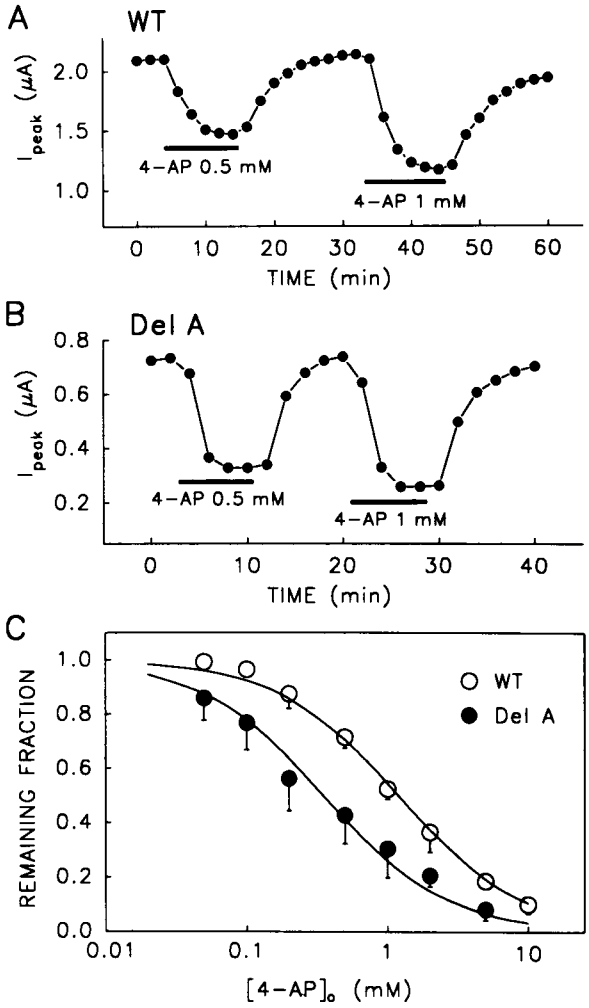


FIGURE 3 Concentration-response relationships of 4-AP block of WT and Del A. (*A* and *B*) Time courses of changes in current amplitudes in representative experiments studying the blocking potency of 4-AP. Currents were induced by depolarization pulses from V_h −80 mV to V_t −20 mV for 500 ms (WT) or 5 s (Del A) once every 2 min. Current amplitudes were measured from leak-subtracted traces as the differences between outward peaks and holding currents. For each of the 4-AP concentrations, the current amplitude measured immediately before adding 4-AP (I_c) and that at the steady-state block by 4-AP (I_d) were measured. Between exposures to 4-AP, the cell was superfused with a 4-AP free solution to allow recovery of current amplitude. (*C*) Relationship between the ratio of I_d to I_c (remaining fraction) and 4-AP concentration. This relationship was fit with the following equation, assuming a 1:1 binding stoichiometry:

$$\text{Remaining fraction} = 1/(1 + [\text{4-AP}]/K_d)$$

where K_d is the apparent 4-AP dissociation constant. Shown are means (symbols, n = 3–7 each) and SD bars (where SD bar is missing, its size is smaller than that of the symbol). The superimposed curves were calculated from the above equation with the best-fit K_d values: 1.20 and 0.35 mM for WT and Del A, respectively.

If the difference in 4-AP's blocking potency between WT and Del A was caused by variation in the rate of channel inactivation, such a difference should be diminished when examined using a voltage clamp protocol that activated both channels to the same degree without causing inactivation in the WT channel. This protocol is shown schematically on the top of Fig. 4. Both WT and Del A were activated by a short-pulse train consisting of 50 depolarization pulses from V_h −80 mV to V_t −20 mV at an interpulse interval of 10 s. The depolarization pulses lasted 12.5 ms, long enough for both currents to approach a plateau without inducing an appreciable decay in the WT current. When this pulse train protocol was applied under the control conditions, the current amplitudes of both WT and Del A were stable (Fig. 4, data labeled as "control"), confirming that the short pulse duration did not induce significant inactivation of WT. After the control data were obtained, the cells were equilibrated with a 4-AP (0.5 mM)-containing solution for 5 min while the membrane voltage was held at −80 mV. The pulse train was then resumed. For both channels, the current amplitude started at a level similar to control and showed a continuous decline during the pulse train, as would be expected if 4-AP blocked the channels in the open state. The time courses of reduction in current amplitudes of WT and Del A were superimposable, indicating that the rate of development and degree of 4-AP block of these two channels were similar under these conditions. The same protocol was applied to four cells expressing WT and four cells expressing Del A from two batches of oocytes. The time course of block development during the pulse train was fit with a single exponential function. The time constants (in number of pulses) were 17.4 and 29.0 for WT and 15.0 and 30.0 for Del A from the first batch of oocytes. For the second batch of oocytes, time constants were 4.0 and 5.0 for WT and 13.0 and 9.6 for Del A. The difference between WT and Del A in the rate of block development was smaller than the variations between batches of oocytes. The mean values of time constants (13.9 ± 11.8 pulses for WT and 16.9 ± 9.0 for Del A) were not statistically different. The degrees of block at the end of the pulse train were 72 ± 6% for WT and 81 ± 8% for Del A ($p > 0.05$). Therefore, unlike the experiments shown in Fig. 1 where 4-AP blocked Del A to a larger degree and at a faster rate than WT, the rate and degree of 4-AP block of these two channels were comparable when studied using the pulse train protocol. These experiments thus suggest that the intrinsic interaction between 4-AP and its binding site in the open channel was similar between WT and Del A. The apparent difference in 4-AP's blocking potency between these two channels when studied using the long depolarization pulse protocol (Figs. 1 and 3) could be explained by the difference in the rate and degree of channel inactivation, which might restrict the access of 4-AP to its binding site (Wagoner and Oxford, 1990; Hice et al., 1992).

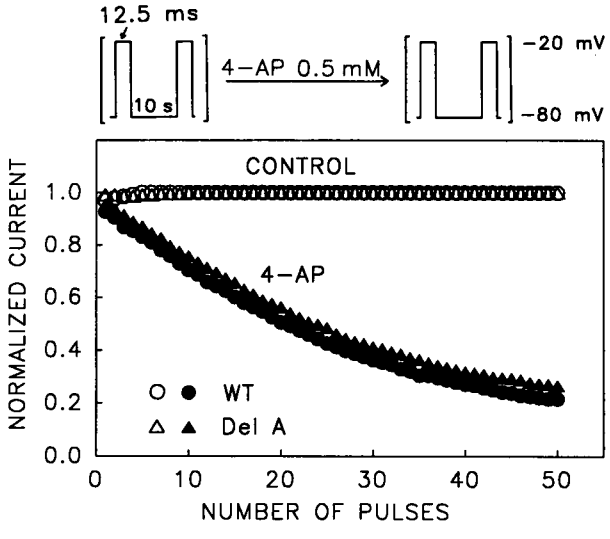


FIGURE 4 Comparison of 4-AP block of WT and Del A using a short-pulse train protocol. The voltage clamp protocol is diagrammed on top. Currents were activated by a train of 50 depolarization pulses from V_h −80 mV to V_t −20 mV at an interpulse interval of 10 s. The duration of the depolarization pulses was 12.5 ms. For each channel, the pulse train was first applied in the absence of 4-AP (control). Then the cell was superfused with a 4-AP (0.5 mM)-containing solution for 5 min during which the membrane voltage was held at −80 mV. Afterwards, the pulse train was resumed. The current amplitudes were measured from leak-subtracted traces as the differences between outward peaks and holding currents, and normalized to the current amplitude induced by the first pulse during the pulse train under the control conditions.

Comparison of 4-AP actions on rKv1.4 between protocols producing no or marked inactivation

To test whether channel inactivation limited the binding of 4-AP to the channel and, thus, reduced its apparent blocking potency, we compared the extent of channel block by 0.5 mM 4-AP produced by the short-pulse train protocol with that produced by the long-pulse protocol. When evaluating the degree of 4-AP block by the long-pulse protocol, the inactivation process of the WT channel prevented a direct assessment of block produced during a prolonged depolarization. Therefore, we used the reduction of peak current amplitude during the second pulse after adding 4-AP to approximate the degree of 4-AP block at the end of the first pulse (V_t −20 mV, duration: 500 ms). Such an approximation is valid because unblock practically did not occur at V_t −80 mV between pulses (see below, Fig. 5). The same amount of depolarization time would be reached by the fortieth pulse during the short-pulse train protocol (V_t −20 mV, total depolarization time: 40 × 12.5 = 500 ms). The current was reduced by only 10 ± 2% after a prolonged depolarization pulse ($n = 4$), much less than that induced by the short-pulse train protocol (68 ± 3%, $n = 4$).

We made a similar comparison for Del A. Here, we compared the degrees of 4-AP (0.5 mM)-induced block by the fortieth pulses during the short-pulse train protocol directly with that produced by the initial 500 ms depolarization during the first pulse in 4-AP (as shown in Fig. 1 *B*). The current

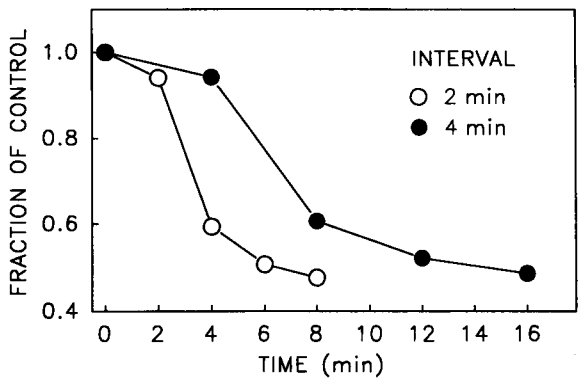


FIGURE 5 Comparison of time courses of development of 4-AP block at different interpulse intervals. Data were from one cell expressing Del A. The currents were activated by 5-s depolarization pulses from V_h −80 mV to V_t −20 mV. The cell was exposed to 4-AP (0.5 mM) twice. The interpulse interval was 2 min during the first exposure, and 4 min during the second exposure. Between exposures, the cell was superfused with a 4-AP free solution to allow recovery from block. For each exposure, the data point at time zero corresponds to current induced by the last pulse before adding 4-AP. Immediately after this pulse, the bath solution was switched to a 4-AP-containing one. The amplitudes of currents induced by the first to fourth pulses in the presence of 4-AP were measured, and normalized to that before adding 4-AP.

was reduced by 76 ± 9% using the pulse train protocol (n = 4), and 54 ± 9% using the long-pulse protocol (n = 6). Therefore, with the same total time of depolarization to the same voltage, 4-AP induced more block when channel inactivation was prevented than when channel inactivation was allowed to occur. The difference was more prominent in WT (that shows a fast inactivation) than in Del (that demonstrates only a slow inactivation).

Comparison of 4-AP block of rKv1.4 at different interpulse intervals

As a final test for the hypothesis that 4-AP blocked rKv1.4 in the open state, we compared the time courses of block development using different interpulse intervals. Fig. 5 shows the time courses of 4-AP block of Del A from one oocyte using a voltage clamp protocol similar to that described for Fig. 1 *B* but with two different interpulse intervals. The degrees of block during the first, second, and fourth pulses in 4-AP were 6, 41, and 52% when tested at an interpulse interval of 2 min. The levels of block at the same pulses were 6, 39, and 51% when the interpulse interval was prolonged to 4 min. Furthermore, the same degree of steady-state block was reached at the fourth pulse in both cases. These results indicated that the degree of block did not depend on how long the cell had been exposed to 4-AP when the membrane was held at −80 mV, but depended on the number of depolarization pulses applied.

State dependence of 4-AP unblock of rKv1.4

After establishing that block of rKv1.4 by 4-AP occurred mainly in the open state, we examined the state dependence of 4-AP unblock. For many open channel blockers, the blocked channels cannot close (deactivate) upon repolarization until they are cleared of bound blockers (Hille, 1991; Colatsky et al., 1990). The unblock event slows the decay of tail currents, and sometimes creates a rising phase of initial tail currents. Neither of these was seen in Del A tail current in the presence of 4-AP (not shown). If the activation gate could close and 4-AP could leave the closed (resting) channel, we expected to see unblock between depolarization pulses when the membrane voltage was held at −80 mV, and the degree of unblock should be increased when the interpulse interval was prolonged. As shown in Fig. 5, the degree of current reduction in 4-AP was the same when examined at an interpulse interval of either 2 min or 4 min. These observations suggest that 4-AP could not leave the channel upon repolarization, but was "trapped" inside the channel by the closure of the activation gate. One possible route by which 4-AP could leave the channel was via the open channel. This predicted a "use-dependent unblock" of 4-AP from the channel. We examined this possibility by studying the effects of changing the interpulse interval on the time course and degree of recovery from 4-AP block after 4-AP was washed out of the bath. The time courses of recovery from 4-AP block were recorded from two oocytes expressing WT rKv1.4 channels. The interpulse interval was 2 min for one and 4 min for the other. The recovery in both cases approached a steady-state by the eighth pulse. The amounts of recovery at the first and second pulses were 6 and 39% when examined at an interpulse interval of 2 min. The corresponding values were 8 and 45% at an interpulse interval of 4 min. Therefore, the time courses of recovery in these two experiments were similar when considered in terms of the number of pulses applied during the recovery period. These observations indicate that 4-AP unblock occurred mainly during depolarization.

We also examined the time course of recovery of Del A from 4-AP block with varying depolarization pulse durations. In these experiments, after the steady state block by 0.5 mM 4-AP was reached, the cells were superfused with a 4-AP-free solution for 5 min while the membrane voltage was held at −80 mV. Very little or no unblock occurred during this interval. Afterwards, a pulse train was initiated which consisted of 90 depolarization pulses from V_h −80 mV to V_t −20 mV at an interpulse interval of 10 s. The pulse duration was 12.5 ms for the first 60 pulses and prolonged to 50 ms for the following 30 pulses. The degree of recovery from 4-AP block was monitored by measuring the increase in current level 12.5 ms after the start of the depolarization pulses. Data summarized from three experiments are shown in Fig. 6. In all three, 12.5-ms pulses induced discernable but slow recovery from 4-AP block. The recovery was markedly accelerated by prolonging the pulse duration to 50 ms. These observations further support the notion that 4-AP unblock mainly occurred through the open state of Del A (and WT rKv1.4) channels.

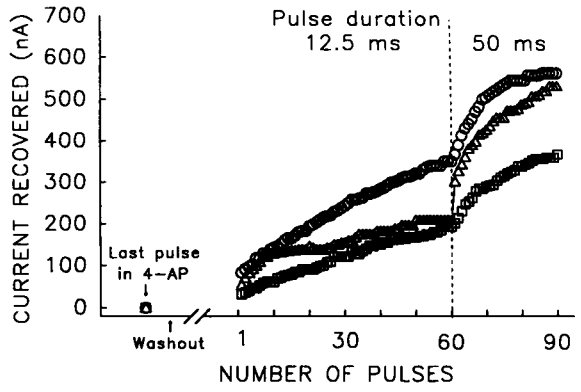


FIGURE 6 Effect of changing the depolarization pulse duration on the time course of Del A recovery from 4-AP block. Shown are data from three experiments denoted by different symbols. The experimental paradigm was the following. After a steady state block by 4-AP (0.5 mM) was reached, the bath solution was switched to a 4-AP free one. The cell was superfused with this solution for 5 min during which the membrane voltage was held at −80 mV (break along x axis). After this washing-out period, a pulse train was initiated that consisted of depolarization pulses from V_h −80 mV to V_t −20 mV applied once every 10 s. The pulse duration was 12.5 ms for the first 60 pulses and prolonged to 50 ms for the next 30 pulses. Current amplitudes 12.5 ms after the start of the depolarization pulses were measured from leak-subtracted traces. The differences between the current amplitudes during the recovery period and the current amplitude at the steady state of 4-AP block were calculated (current recovered) and plotted against pulse number during the pulse train.

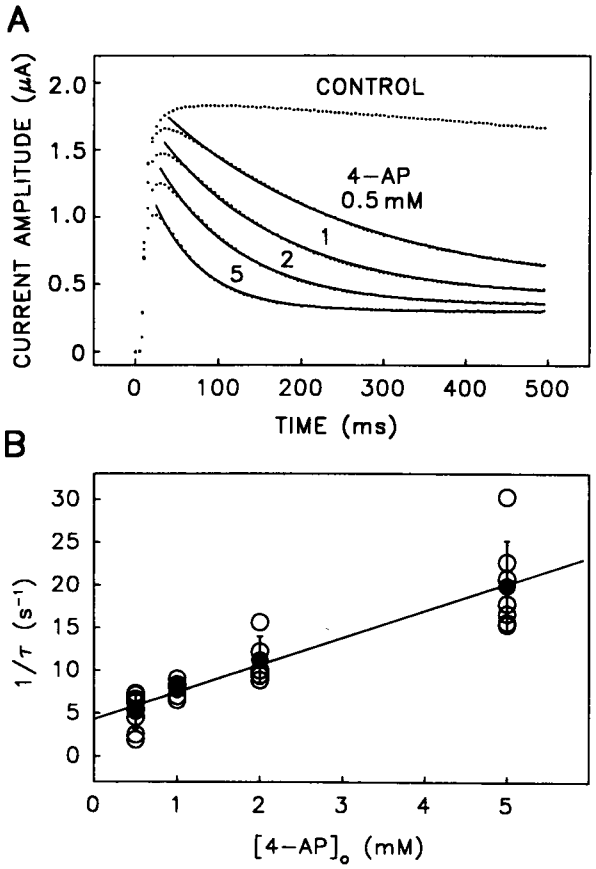


FIGURE 7 Kinetics of interaction between 4-AP and Del A. (*A*) Concentration-dependent acceleration of decay of Del A by 4-AP. Data were obtained from one cell that was given four exposures of 4-AP at increasing concentrations (0.5, 1.2, and 5 mM). Between exposures, the cell was superfused with a 4-AP free solution to allow recovery. The currents were activated by depolarization pulses from V_h −80 mV to V_t −20 mV. Shown are leak-subtracted traces recorded before 4-AP exposure (control) and during the first pulses after adding 4-AP (concentrations marked). Curves superimposed on the current traces were calculated from a single exponential function with the best-fit time constants: 225, 146, 98, and 58 ms for [4-AP] of 0.5, 1, 2, and 5 mM, respectively (*B*) Estimation of the apparent binding and unbinding rate constants of 4-AP. The rate of block development (reciprocal of time constant of block development, as estimated in *A*) is plotted against 4-AP concentration. Shown are data from individual experiments (○, 5–7 for each 4-AP concentration), with means (●) and SD bars. The superimposed regression line has a slope of 3.2 mM^{-1} s^{-1} and a y axis intercept of 4.3 s^{-1}.

Kinetics of interactions between 4-AP and Del A

The above observations suggest that block and unblock of 4-AP mainly occurred via the open state of rKv1.4 (WT and Del A). Therefore, the interaction between 4-AP and the channels can be described by the following reaction:

$$O \underset{K_{BO}}{\overset{4\text{-}AP_n\ K_{OB}}{\rightleftharpoons}} B$$

where "O" and "B" denote channels' open state free of 4-AP and blocked by 4-AP, respectively, K_{OB} and K_{BO} are the binding and unbinding rate constants, and n is the number of 4-AP molecules needed to block one channel. Because the activation of Del A was much faster, and its decay (inactivation) was much slower, than the rate of current reduction induced by 4-AP (Figs. 1 *B* and 2), the decay time course of Del A in the presence of 4-AP was used to estimate the kinetic parameters of 4-AP binding and unbinding.

Fig. 7 *A* compares the time courses of Del A immediately before and during the first pulses after adding 4-AP of several concentrations. 4-AP induced a marked decay of Del A in a concentration-dependent manner. The 4-AP-induced decay of Del A followed a single exponential time course (Fig. 7 *A*), supporting a 1:1 binding stoichiometry. Such a stoichiometry further predicts the following relationship between the rate of block development and the binding and unbinding rate constants:

$$\text{Rate of block} = 1/\tau = K_{OB}[\text{4-AP}] + K_{BO}.$$

K_{OB} and K_{BO} could be estimated from the slope and the y axis intercept of the line relating 4-AP concentration to the rate of block development. Fig. 7 *B* depicts data summarized from multiple experiments along with mean values. The regression line generated an apparent K_{OB} of 3.2 mM^{-1} s^{-1} and K_{BO} of 4.3 s^{-1}. These rate constants indicate that 4-AP blocks rKv1.4 with slow kinetics, similar to 4-AP's actions on I_A in GH_3 cells (Wagoner and Oxford, 1990) and I_K in B-lymphocytes (Choquet and Korn, 1992).

Sidedness of 4-AP actions on rKv1.4

To understand the mechanism of 4-AP actions, it is important to know from which side of the cell membrane 4-AP exerted

its actions. It has been shown for many K channels that 4-AP block occurs from the intracellular surface of the membrane (Kirsch and Narahashi, 1983; Choquet and Korn, 1992; Davies et al., 1991; Kirsch and Drewe, 1993). To test whether this is also the case for rKv1.4, we injected 4-AP directly into the cytoplasm and compared the resulting changes in Del A with those after external application. In these experiments, the cell volumes were estimated by measuring the cell diameters under the microscope using a micrometer and assuming that oocytes were spheres. The amounts of 4-AP injected (10–23 nl of 20 mM 4-AP solution) were adjusted to reach a cytoplasmic concentration of 0.5 mM (ignoring the amount of 4-AP that leaked out of the cells). Insertion of the injecting microelectrode induced negligible or only small and transient changes in the holding current and Del A. Furthermore, injection of water (30 nl, $n = 2$) did not induce any appreciable changes in Del A. Intracellular injection of 4-AP blocked the channels potently ($n = 4$). Fig. 8 shows data from one such experiment. The current induced by the first pulse after 4-AP injection had a much reduced peak amplitude and a very fast decay time course. The second pulse induced an even smaller current than the first pulse. However, the time course of the current during the second pulse was biphasic: after the decay during the initial 300 ms, the current increased slowly for the remaining period of the depolarization. The currents induced by the third and subsequent pulses after 4-AP injection showed a further gradual recovery (data not shown), suggesting that 4-AP continuously leaked out of the cell leading to block reversal. The qualitative similarity in the effects of 4-AP on Del A after intracellular injection and external application indicates that 4-AP blocked rKv1.4 from inside.

Effects of 4-AP on single channel properties of Del A

To assess directly the kinetics of 4-AP actions, we examined its effects (0.5 or 1 mM) on single channel currents of Del A recorded from cell-attached patches. 4-AP was added to the bath solution, because it could permeate the cell membrane and worked from inside (Fig. 8). To facilitate measurements of single channel currents, we used V_t of +40 mV. Fig. 9 *A* illustrates Del A single channel currents recorded from the same patch before and after adding 0.5 mM 4-AP to the bath solution. Under the control conditions, upon depolarization Del A channels opened with a short latency and displayed bursting activities. When Del A channel activities were seen, they generally lasted throughout the duration of

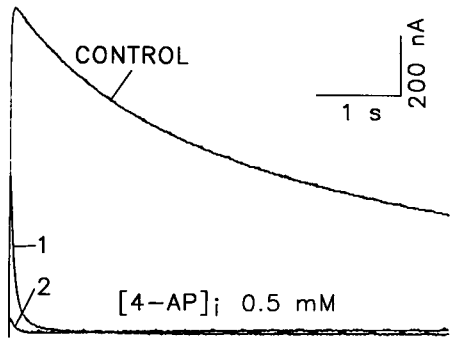


FIGURE 8 Effects of intracellular injection of 4-AP on Del A. The currents were activated by 5 s depolarization pulses from V_h −80 mV to V_t −20 mV once every 2 min. The current amplitude was stable under the control conditions (traces marked by "control"). Then, 13 nl of 20 mM 4-AP solution was injected into the cytoplasm (estimated $[4\text{-AP}]_i$ = 0.5 mM). Current traces marked by "1" and "2" were activated by the first and second pulses after 4-AP injection.

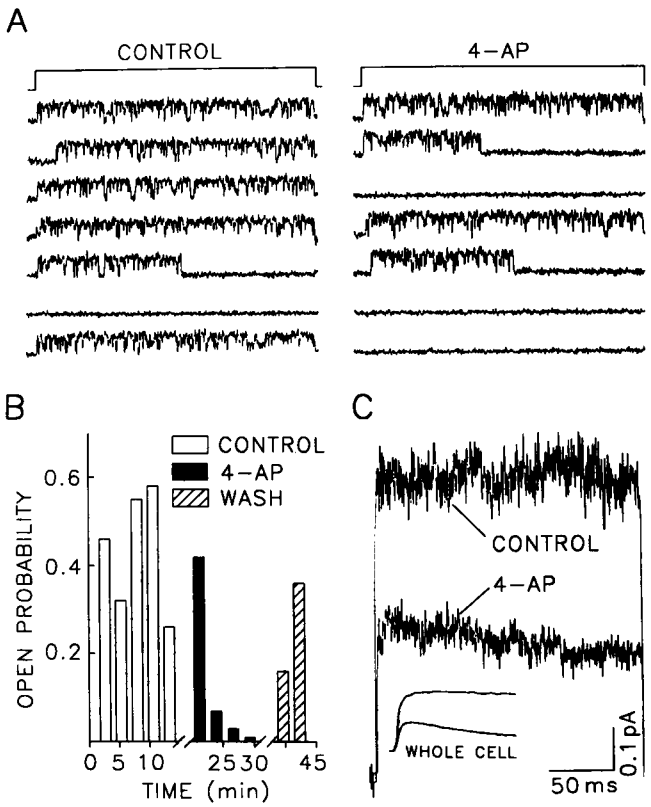


FIGURE 9 Effects of 4-AP on single channel properties of Del A. (*A*) Representative current traces recorded before (*left*) and after (*right*) adding 0.5 mM 4-AP to the bath solution. Single channel currents were recorded from a cell-attached patch. The voltage clamp protocol is diagrammed above the current traces: 200 ms depolarization pulses from V_h −80 mV to V_t +40 mV applied every 10 s. (*B*) Time course of changes in channel open probability during the same experiment as shown in *A*. The open probability (P_o) was calculated according to the following relationship:

$$P_o = I/iN$$

where I was peak amplitude of mean current averaged over 16 consecutive leak-subtracted traces; i was the mean single channel current amplitude (0.71 pA); N was the number of channels in the patch ($n = 2$). During washing in and washing out of 4-AP, a superfusion period of 5 min was allowed during which the patch membrane voltage was held at −80 mV (breaks along the x axis). (*C*) Effects of 4-AP on the ensemble average of Del A and a comparison with those on whole-cell Del A current. The ensembles were averaged from 112 control traces and 66 traces in the presence of 4-AP. (*inset*) Whole-cell currents recorded before (larger amplitude) and after adding 0.5 mM 4-AP to the bath solution. Data were obtained from another cell. The currents were activated by pulses from V_h −80 mV to V_t −20 mV. Both ensemble averages and whole-cell currents represent data recorded during the first 200 ms after the start of depolarization.

the depolarization pulses. Occasionally, bursts stopped before the end of a pulse and the channel entered a long-lasting closed state, or channels failed to open at all during the entire depolarization pulse (e.g., the fifth and sixth sweeps in the left panel of Fig. 9 *A*, respectively). In the presence of 4-AP, the most noticeable change was an increase in the frequency of sweeps without openings. Moreover, sweeps with bursts terminated before the end of the depolarization pulses occurred more often. The time course of changes in the open probability of Del A in the same patch before, during, and after exposure to 4-AP is plotted in Fig. 9 *B*. In the presence of 4-AP, the channel open probability was initially comparable to control but then showed a continuous decline after repetitive depolarizations. The open probability was greatly reduced after a 10-min continuous pulsing. After washing out of 4-AP, the channel open probability recovered in a time-dependent manner. Fig. 9 *C* compares the ensemble averages of Del A under the control conditions and after the open probability had been reduced by 4-AP. They show characteristics similar to those of whole-cell recordings (*inset* of Fig. 9 *C*), although the degree of block in whole-cell recording (V_t −20 mV) seems to be higher than that in single channel recording (V_t +40 mV) (see below, Fig. 10).

The effects of 4-AP on single channel properties of Del A are summarized in Table 1. 4-AP did not alter the single channel current amplitude. Its effects on the burst kinetics of Del A, if any, were modest. The mean burst duration was shortened, likely because of a small decrease in the mean number of openings per burst. The mean open time was not altered by 4-AP, whereas the mean closed time within bursts appeared to be slightly prolonged. Therefore, single channel analysis of 4-AP's actions on Del A are consistent with conclusion from whole-cell recordings: the blocking and unblocking rates of 4-AP are slow so that it could not compete with the fast closing and opening transitions constituting bursts. 4-AP's blocking effects manifested as a shortening of burst duration and more importantly, a reduction of channel open probability.

Voltage dependence of 4-AP actions on Del A

Effects of varying depolarization voltage on 4-AP's blocking potency

There were two reasons for us to speculate that changing the voltage of depolarization pulses would affect the blocking potency of 4-AP. First, stronger depolarizations could increase the degree of channel activation, which in turn might enhance the effects of an open channel blocker such as 4-AP (Snyders et al., 1992b). Second, if 4-AP blocked rKv1.4 in its protonated form from inside (Choquet and Korn, 1992; Kirsch and Narahashi, 1983) by binding to a site within the membrane electrical field, stronger depolarizations might enhance 4-AP's blocking potency by an electrophoresis action (Woodhull, 1973; Snyders et al., 1992b). Therefore, we recorded Del A at different V_t before and after adding 4-AP. In these experiments, the current recordings at each V_t were repeated 3–4 times at an interpulse interval of 1 min in the presence of 4-AP

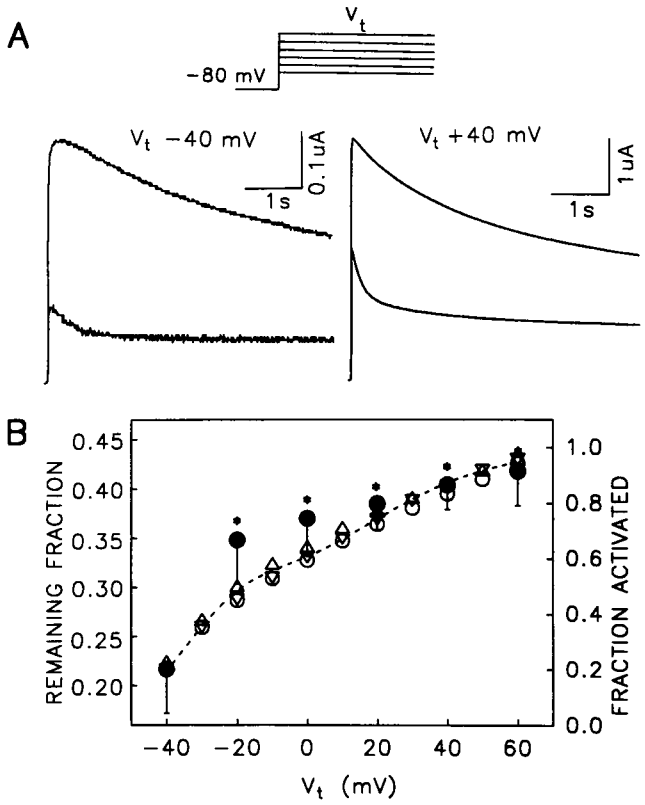


FIGURE 10 Effects of changing depolarization voltage on the degree of Del A block by 4-AP. Currents were activated by 5 s depolarization pulses from V_h −80 mV to various V_t (*inset* of *A*) at an interpulse interval of 1 min under the control conditions, and at the steady state of block by 4-AP (0.5 mM). (*A*) Comparison of degrees of Del A block by 4-AP at V_t −40 and +40 mV in one cell. For each V_t, the leak-subtracted control current (larger amplitude) and the current in the presence of 4-AP are superimposed. To facilitate comparison, the control traces are scaled so that their peak amplitudes match each other. The current amplitude at the end of the pulse was reduced by 72% at −40 mV and 56% at +40 mV. (*B*) Data summary of degrees of Del A block by 4-AP at various V_t and comparison with the voltage dependence of Del A activation. The remaining fraction of current at the steady state of 4-AP block (●, *left y* axis) is plotted against V_t, along with SD bars (n = 3,*: $p < 0.001$ relative to data at −40 mV by unpaired t-test). The voltage dependence of activation of Del A was determined by calculating the peak chord conductances at various depolarization voltages (peak current amplitude divided by the driving force, assuming the reversal potential to be −100 mV), and normalizing these values by that measured at +70 mV. The relationship between voltage and the normalized peak chord conductance was fit with the following equation (Tseng and Tseng-Crank, 1992):

$$\text{Normalized conductance} = A_1/(1 + \exp[(V_1 - V_t)/k_1]) + (1 - A_1)/(1 + \exp[(V_2 - V_t)/k_2])$$

Data from individual experiments are depicted by open symbols (different symbols represent different experiments, n = 3). The average activation curve is shown as a dotted curve (right y axis), and was calculated from the above equation with the following parameter values: A_1 = 0.46, V_1 = −36.4 mV, k_1 = 6.8 mV, V_2 = 18.0 mV, and k_2 = 18.6 mV.

to ensure that 4-AP block had reached a steady state at that particular V_t. Fig. 10 *A* compares the degree of 4-AP block of Del A at −40 and +40 mV in one cell. The current measured at the end of the 5-s pulse was reduced more at the less

TABLE 1 Effects of 4-AP on single-channel properties of Del A

	Control (2)	4-AP 0.5 mM (2)	Control (4)	4-AP 1 mM (4)
Current amplitude (pA)	0.77	0.80	0.76 ± 0.04	0.76 ± 0.06
Mean open time (ms)	2.04	2.06	2.19 ± 0.26	2.09 ± 0.16
Mean closed time (ms)	0.29	0.30	0.29 ± 0.04	0.35 ± 0.04*
Mean burst duration (ms)	35.1	30.8	39.0 ± 6.8	29.1 ± 8.1**
Mean number of openings per burst	13.8	12.8	14.1 ± 2.4	10.5 ± 2.5*

Values in parentheses are numbers of patches studied; shown are average values (for [4-AP] = 0.5 mM) or mean ± SD (for [4-AP] = 1 mM); see Materials and Methods for determination of single-channel parameters; statistical significance was tested by paired *t*-test; * $p < 0.05$ and ** $p < 0.01$, relative to control.

positive voltage (−40 mV, 72%) than at the more positive voltage (+40 mV, 56%). This was a consistent finding from three experiments over a voltage range from −40 to +60 mV (Fig. 10 *B*). Therefore, in contrast to our prediction, stronger depolarization reduced the potency of steady state 4-AP block of Del A. The depolarization dependence of reduction in 4-AP's blocking potency is somewhat similar to the voltage dependence of Del A activation (Tseng-Crank et al., 1993) (Fig. 10 *B*): for both, the most steep voltage dependence occurred in the voltage range negative to −20 mV, with a more modest voltage dependence in the more positive voltage range. This similarity suggests that channel conformational changes accompanying activation can lead to a reduction in the 4-AP binding affinity (McCormack et al., 1994).

Effects of varying holding voltage on 4-AP's action

If voltage could modulate binding site affinity as suggested by Fig. 10, we should be able to demonstrate depolarization-induced "unblock" using a suitable voltage clamp protocol. Therefore, exposing a cell to 4-AP while holding the membrane voltage above the activation threshold (−55 to −50 mV) should induce 4-AP block during the interpulse interval, and unblock should occur after a stronger depolarization. Fig. 11 illustrates the effects of 4-AP on Del A studied at V_h −50 and −40 mV (*middle* and *right* panels), and compares them with those at V_h −80 mV (*left* panel). Holding the membrane voltage at −40 mV induced an outward shift in current level, which was sensitive to 4-AP (note the 4-AP induced inward shift in holding current at V_h −40 mV). These observations indicate that Del A channels were activated at V_h −40 mV, and were blocked by 4-AP before the first depolarization pulse. The first pulse induced a slowly rising outward current, whose time course was drastically different from that of current induced by the first pulse in 4-AP recorded at V_h −80 mV. This time course suggests unblock of 4-AP upon depolarization from −40 to −20 mV. The current amplitudes were measured as the differences between current levels at the end of the 5-s pulses and the holding currents. The current amplitude at the end of the first pulse in 4-AP was actually larger than that under the control conditions. Therefore, if the two traces were aligned by the holding currents, there would be a "cross-over" of the two traces, similar to the phenomenon described for the "resting-state block" mode of 4-AP actions (Campbell et al., 1993; Castle and Slawsky, 1993; Yeh et al., 1976). At V_h −50 mV, the degree of channel

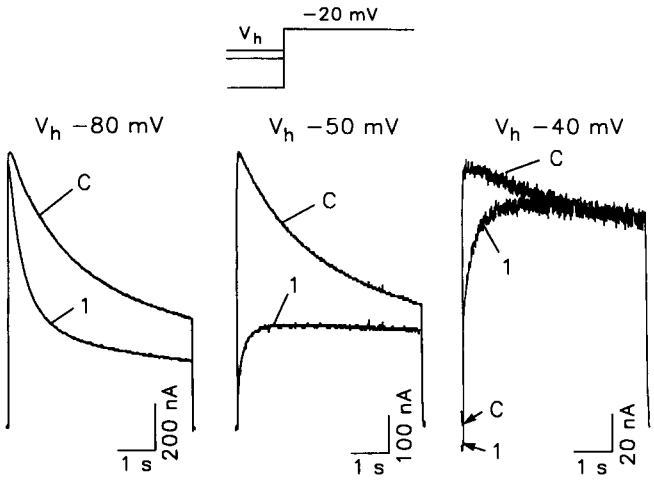


FIGURE 11 Effects of changing holding voltage on 4-AP actions on Del A. The currents were activated by 5 s depolarization pulses to V_t −20 mV from V_h of −80, −50, or −40 mV (*inset*). For each V_h, the leak-subtracted current recorded under the control conditions (marked by "C") and that induced by the first pulse after adding 0.5 mM 4-AP (marked by "1") are superimposed. To facilitate comparison, the gain was adjusted so that the peak amplitudes of the control traces match each other. For V_h −40 mV, the holding current levels are also marked to indicate the 4-AP-induced inward shift of holding current. Note that at V_h −40 mV, although the current levels at the end of the 5-s-pulses were superimposable, the actual difference between this current level and the holding current is larger during the first pulse in 4-AP than in control.

activation was very small. Adding 4-AP did not induce an appreciable inward shift in the holding current as observed at V_h −40 mV. However, the first depolarization pulse still induced a slowly rising outward current, suggesting 4-AP unblock at −20 mV.

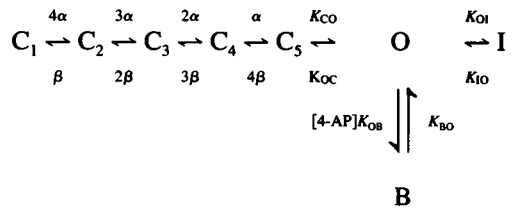
DISCUSSION

State-, voltage-, and time dependences of 4-AP actions on rKv1.4

Our data show that development of 4-AP block of rKv1.4 required channel opening (Figs. 1, 2, and 5). Furthermore, upon repolarization 4-AP was trapped inside the channel, and unblock also occurred via the open channel (Fig. 6). These observations suggest that "closure" of the activation gate limits 4-AP's access to and exit from its binding site. Removing the fast inactivation gate of rKv1.4 increased the apparent potency of 4-AP action (Fig. 3). A short-pulse train

that activated channels without inactivation induced more 4-AP block than a long-pulse that inactivated channels. Therefore, "closure" of inactivation gate also hinders 4-AP binding to rKv1.4. After establishing 4-AP block at V_h −40 mV, further depolarization led to a slow unblock. Under this condition, the current trace recorded in the presence of 4-AP crossed over the control trace, suggesting that 4-AP-bound channels could not inactivate (Fig. 11). Therefore, channel inactivation and 4-AP binding are mutually exclusive. The unblock phenomenon described above and the observation that the degree of steady state 4-AP block became smaller at more positive voltages (Fig. 10) suggest that depolarization can reduce the affinity of the channel binding site for 4-AP, possibly by inducing conformational changes in the channel protein (McCormack et al., 1994). Therefore, depolarization might have a dual effect on 4-AP action: regulating 4-AP access to its binding site by controlling channel gating, and modulating 4-AP binding affinity by controlling the conformation of the binding site.

We approximated the time course of 4-AP block development by the time course of decay of Del A induced by the first pulse after adding 4-AP (Fig. 7). In the literature, other approaches have been described that are used to estimate the binding and unbinding kinetics of open channel blockers. For example, the time course of block development has been approximated by: (1) the time course of changes in the ratio of current in the presence of a blocker (I_d) to the control current (I_c) (Duan et al., 1993), (2) the time course of changes in a difference current obtained by subtracting I_d from I_c (Wagoner and Oxford, 1990), or (3) the fast component of a biexponential fit to I_d (Snyders et al., 1992a). To compare our analysis with the other approaches, we simulated the control Del A current and currents induced by the first pulse after exposure to 4-AP of different concentrations, and compared the results of different methods of analysis. The simulation was carried out using a fourth-order Runge-Kutta routine (QuickPak Scientific, Crescent Software, Inc., Ridgefield, CT), based on the following state diagram (Zagotta and Aldrich, 1990; Tseng-Crank et al., 1993).



$$\alpha = 230\exp(V/70.3)\ (s^{-1}) \qquad \beta = 20\exp(-V/14.6)\ (s^{-1})$$

C_i (i = 1–5), O, and I are closed, open, and inactivated states of the channel, B is the 4-AP-blocked open state, and K_{ij} denotes the transition rate constant from state i to state j. For the simulation, K_{CO} (3125 s^{-1}) and K_{OC} (503 s^{-1}) were taken from our previous study (Tseng-Crank et al., 1993). These values are similar to those calculated from the single channel parameters here (see below). K_{OB} (3.2 mM^{-1} s^{-1}) and K_{BO} (4.3 s^{-1}) were taken from whole-cell current measurements (Fig. 7). To reproduce the time course of whole-cell Del A current, K_{OI} and K_{IO} were assigned the values of 1.2 and 1.1 s^{-1}, respectively. The values of K_{OB} (mM^{-1} s^{-1}), K_{BO} (s^{-1}) and K_d(mM) estimated by our method were 1.6, 3.5, and 2.1. The corresponding values were 1.4, 5.8, and 4.1 by the first method described above (Duan et al., 1993), 1.1, 6.2, and 5.6 by the second method (Wagoner and Oxford, 1990), and 1.5, 4.8, and 3.2 by the third method (Snyders et al., 1992a). Therefore, all four methods generated parameter values different from the "true" values, with our estimation of K_d slightly better than the other three. We conclude from the results of simulation that the K_{OB} value we estimated from whole-cell data probably was underestimated. This can probably explain why the K_d value calculated from K_{BO}/K_{OB} (1.4 mM) is higher than that estimated from the concentration-response relationship (0.35 mM, Fig. 3).

Another way to evaluate the analysis of kinetics of 4-AP binding and unbinding is to compare data from whole-cell currents with those from single-channel recordings. Based on the above state diagram and assuming that bursts constituted transitions between C_5 and O (Zagotta and Aldrich, 1990; Tseng-Crank et al., 1993), the mean open time (t_o), mean closed time within bursts (t_c), mean burst duration (D_b), and mean number of openings per burst (m_r) should have the following relationships with the rate constants of channel gating under the control conditions (Colquhoun and Hawkes, 1983):

$$t_c = 1/K_{CO}; \quad t_o = 1/(K_{OC} + K_{OI})$$

$$D_b = [1 + (K_{OC}/K_{CO})]/K_{OI}; \quad m_r = 1 + (K_{OC}/K_{OI})$$

In the presence of 4-AP, t_c should not be altered, and the other parameters should behave as the following:

$$t_o' = 1/(K_{OC} + K_{OI} + [4\text{-AP}]K_{OB})$$

$$D_b' = [1 + (K_{OC}/K_{CO})]/(K_{OI} + [4\text{-AP}]K_{OB});$$

$$m_r' = 1 + [K_{OC}/(K_{OI} + [4\text{-AP}]K_{OB})]$$

From control values of t_o, t_c, and D_b (Table 1), the following rate constants were calculated according to the above equations: K_{CO} = 3450 s^{-1}, K_{OC} = 420 s^{-1}, and K_{OI} = 30 s^{-1}. With K_{BO} estimated from whole-cell data (4.3 s^{-1}) and K_{OB} calculated from K_{BO}/K_d (12.3 s^{-1}), at 1 mM 4-AP the calculated values of t_o', D_b' and m_r' are 2.1, 25.1, and 10.5 ms, respectively. These values are the same or similar to corresponding experimental measurements (2.1, 29.1, and 10.5 ms, Table 1), even though the depolarization voltage was −20 mV in whole-cell recordings but +40 mV in single-channel recordings. Therefore, both whole-cell and single-channel recordings indicate that 4-AP is a slow blocker of rKv1.4. At the single channel level, 4-AP's blocking actions manifested as a shortening of burst durations and more frequent termination of burst activities before the end of depolarization pulses. These correspond to the accelerated decay of whole-cell current. 4-AP also reduced the likelihood that channel could be opened by depolarization, corresponding to the reduction of peak current amplitude seen at the whole-cell level.

There was a slight prolongation of mean closed time within bursts (t_c) in the presence of 4-AP that is not consistent with the above scheme. This may be because of the method used in the estimation of t_c. We fit the closed time distribution with a double exponential function and assigned the shorter time constant to be equal to t_c. In the presence of 4-AP, a prolongation of interburst intervals caused by channel block might cause an overestimation of t_c.

Because 4-AP could not dissociate from the channel between depolarization pulses and unblock occurred mainly in the open state, unblock during depolarization should manifest as a delay in the latency to first opening. Indeed, this has been observed for 4-AP block of kv3.1 (Kirsch, and Drewe, 1993). However, we did not observe a consistent and significant delay in the first latency in the presence of 4-AP. Assuming that the time constant of unblock was 232 ms ($1/K_{BO}$, where K_{BO} is 4.3 s^{-1}), the 200-ms pulses we used during single channel recordings would allow us to see unblock from a single channel 58% of the time. However, because the patches we studied contained multiple channels, normal openings from unblocked channels in the patch might have masked these late openings.

Comparison with previous observations and structural implications

The state- and voltage-dependences of 4-AP actions on rKv1.4 are similar to those described for 4-AP actions on Kv2.1 and Kv3.1 (Kirsch and Drewe, 1993) and on a *Shaker* (29-4) channel (McCormack et al., 1994). In all these channels, 4-AP block and unblock occur mainly in the open state. For Kv2.1 (Kirsch et al., 1993) and *Shaker* 29-4 channel (McCormack et al., 1994), 4-AP's blocking potency is clearly reduced by depolarization to voltages above the threshold of channel activation, similar to the observations reported here on rKv1.4. At the single channel level, 4-AP reduced the burst duration and increased the percentage of null sweeps without affecting the single channel current amplitude of *Shaker* 29-4, Kv3.1, and Kv2.1 (McCormack et al., 1994; Kirsch and Drewe, 1993). These again are similar to the effects of 4-AP on rKv1.4 single channel currents.

Although rKv1.4 was cloned from a rat heart library, the actions of 4-AP on rKv1.4 (open-state block) are qualitatively different from those on the native I_A in rat ventricular myocytes (resting-state block) (Castle and Slawsky, 1993). This suggests that rKv1.4 is not equivalent to the native I_A in rat heart. A K channel clone belonging to another (*shal*-related) gene family has been isolated from and identified in rat heart (Blair et al., 1991; Baldwin et al., 1991). This K channel has fast-activating and -inactivating kinetics resembling the native I_A. However, the K_d value for 4-AP block of this K channel clone is $\geq$5 mM, much higher than that for 4-AP block of the native I_A (0.2 mM). Another explanation for the discrepancy in 4-AP actions between K channel clones and native I_A is that native I_A might be a heteromultimer (Po et al., 1993), and different subunit composition might influence channel pharmacology qualitatively (Po et al., 1993; Welling et al., 1993).

Although 4-AP binding to rKv1.4 required channel opening, stronger depolarizations reduced the degree of 4-AP block at the steady state. Furthermore, the depolarization-induced reduction in 4-AP block paralleled the voltage dependence of channel activation. Channel opening during depolarization is a complicated and not well understood process. It might involve voltage-dependent conformational changes (possibly caused by movements of S4 domains of four subunits (Liman et al., 1991; Papazian et al., 1991)), with a final opening transition that displays little or no voltage dependence (Zagotta and Aldrich, 1990) (possibly involving the "leucine-zipper" domains (McCormack et al., 1991)). One possible explanation for the dual effect of membrane depolarization on 4-AP binding is that S4 domain movements expose the 4-AP binding site, whereas the final opening transition lowers the binding site affinity. This hypothesis will be consistent with the observations on mutant Kv2.1 and Kv3.1 chimera channels showing a quantitative correlation between an increase in the stability of open conformations of K channels and a reduction in 4-AP blocking potency (Kirsch et al., 1993). This might also explain the observation that a mutation in the leucine-zipper domain that interferes with the coupling between S4 domain movements and channel opening (Schoppa et al., 1992) increases the channel's 4-AP sensitivity (McCormack et al., 1991). If 4-AP interacts with rKv1.4 as suggested, this scheme will be similar to that proposed for 4-AP actions on I_{KDR} of squid axon (Kirsch et al., 1986). Here, 4-AP binding is slow at highly hyperpolarized voltages but accelerated by moderate depolarizations, as if 4-AP binding occurs in an intermediate state in the activation pathway. However, channel opening leads to 4-AP dissociation from the channel. A better understanding of the mechanism underlying the state- and voltage-dependence of 4-AP actions on various K channels might provide insights into not only how K channel blockers interact with their target channels, but also the relationship between S4 domain movements and channel opening.

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