

## Mechanism of 4-Aminopyridine Action on Voltage-gated Potassium Channels in Lymphocytes

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**ABSTRACT** The mechanism by which 4-aminopyridine (4-AP) blocks the delayed rectifier type potassium ( $K^+$ ) channels present on lipopolysaccharide-activated murine B lymphocytes was investigated using whole-cell and single channel patch-clamp recordings. 4-AP (1  $\mu$ M–5 mM) was superfused for 3–4 min before applying depolarizing pulses to activate the channel. During the first pulse after application of 4-AP above 50  $\mu$ M, the current inactivated faster, as compared with the control, but its peak was only reduced at high concentrations of 4-AP ( $K_d = 3.1$  mM). During subsequent pulses, the peak current was decreased ( $K_d = 120$   $\mu$ M), but the inactivation rate was slower than in the control, a feature that could be explained by a slow unblocking process. After washing out the drug, the current elicited by the first voltage step was still markedly reduced, as compared with the control one, and displayed very slow activation and inactivation kinetics; this suggests that the  $K^+$  channels move from a blocked to an unblocked state slowly during the depolarizing pulse. These results show that 4-AP blocks  $K^+$  channels in their open state and that the drug remains trapped in the channel once it is closed. On the basis of the analysis of the current kinetics during unblocking, we suggest that two pathways lead from the blocked to the unblocked states. Computer simulations were used to investigate the mechanism of action of 4-AP. The simulations suggest that 4-AP must bind to both an open and a nonconducting state of the channel. It is postulated that the latter is either the inactivated channel or a site on closed channels only accessible to the drug once the cell has been depolarized. Using inside- and outside-out patch recordings, we found that 4-AP only blocks channels from the intracellular side of the membrane and acts by reducing the mean burst time. 4-AP is a weak base ( $pK = 9$ ), and thus exists in ionized or nonionized form. Since the  $K_d$  of channel block depends on both internal and external pH, we suggest that 4-AP crosses the membrane in its nonionized form and acts from inside the cell in its ionized form.

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## INTRODUCTION

The drug 4-aminopyridine (4-AP) is known to block voltage-activated  $K^+$  channels expressed in a variety of cell types including neurons (Pelhate and Pichon, 1974; Ulbricht and Wagner, 1976) and muscle (Gillespie and Hutter, 1975). Cells of the immune system, such as B and T lymphocytes and macrophages, express voltage-activated  $K^+$  channels also sensitive to 4-AP (references in Choquet and Korn, 1988a; Gallin, 1988; Lewis and Cahalan, 1990). In addition, 4-AP blocks different lymphocytic functions, including mitogen-induced cell proliferation (DeCoursey, Chandy, Gupta, and Cahalan, 1984; Amigorena, Choquet, Teillaud, Korn, and Fridman, 1990) and killing by natural killer (NK) cells and cytotoxic T cells (reviewed in Lewis and Cahalan, 1990). However, the concentration of 4-AP required to inhibit cell functions may be 5- to 10-fold higher than that required to block  $K^+$  channels during voltage clamp experiments (Chandy, DeCoursey, Cahalan, McLaughlin, and Gupta, 1984; Schlichter, Sidell, and Hagiwara, 1986; Amigorena et al., 1990). Part of this effect may result from a decrease in the channel-blocking potency of 4-AP in the presence of serum contained in the culture medium (Price, Lee, and Deutsch, 1989). Alternatively, the discrepancy may be due to the voltage-dependent properties of 4-AP binding, observed in experiments on nerve and muscle (Chandy et al., 1984; Schlichter et al., 1986). To test this hypothesis directly, we investigated the mechanism of action of 4-AP on lipopolysaccharide (LPS)-activated murine B lymphocytes, since these cells express mainly a single type of voltage-activated  $K^+$  channel (Choquet, Sarthou, Primi, Cazenave, and Korn, 1987).

Previous studies have suggested that 4-AP binds to closed channels, is released from open channels when the cell is depolarized, and interferes with inactivation (Yeh, Oxford, Wu, and Narahashi, 1976; Thompson, 1982; Simurda, Simurdova, and Christé, 1989; Kehl, 1990). On the basis of experimental data and computer simulations, we conclude that 4-AP blocks lymphocyte  $K^+$  channels when they are in their open state. Furthermore, the drug remains trapped in the channels at hyperpolarized potentials, and relief of block only occurs upon cell depolarization. Similar results have recently been obtained on the delayed rectifier  $K^+$  channels in GH3 cells (Wagoner and Oxford, 1990).

It has also been shown that 4-AP blocks  $K^+$  channels when applied outside or inside the cell (Yeh et al., 1976; Hermann and Gorman, 1981; Wagoner and Oxford, 1990). We found that in isolated patches of membrane, the  $K^+$  channels are blocked only if the drug is present on the cytoplasmic side. Finally, demonstration of a pH dependence of 4-AP actions led us to propose that the drug crosses the membrane in its nonionized form, and that it blocks the channels from the inside once reionized.

A preliminary report of this work has appeared in abstract form (Choquet and Korn, 1990).

## METHODS

*Cell Isolation and Culture*

LPS-activated B lymphocytes were obtained and cultured as previously described (Amigorena et al., 1990). Briefly, spleen cell suspensions, obtained from 8-wk-old B6.D2(H-2b,d) mice (Institut Pasteur, Paris, France) were depleted of T lymphocytes by treatment with a combina-

tion of rat anti-Thy 1 mAb (42.21 and J1j.10) for 30 min at 20°C in Hank's balanced salt solution (Gibco BRL, Paisley, Scotland), followed by incubation with 1:40 diluted rabbit complement (twice for 15 min at 37°C). Resting B cells were prepared by Percoll (Pharmacia LKB Biotechnology, Bois d'Arcy, France) density centrifugation. Cells banding between 70 and 60% Percoll were collected and usually represented 60–80% of the loaded population. They were 95% IgM positive (as detected by cytofluorometric analysis), did not respond to the T cell mitogen concanavalin A (Sigma Chemical Co., St Louis, MO), and had a mean volume of  $\sim 120 \mu\text{m}^3$  (Amigorena et al., 1990). They represented the source of small resting B lymphocytes.  $2\text{--}3 \times 10^5$  resting B cells per well were cultured in flat-bottomed 24-well Falcon culture plates (Becton Dickinson, Ehemboegem, Belgium) in 0.5 ml of RPMI 1640 (Gibco) containing: 25 mM HEPES, 1 mM L-glutamine, 100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin,  $5 \cdot 10^{-5}$  M 2-mercaptoethanol, 10% heat-inactivated FCS (Jacques Boy, Reims, France), and 10  $\mu\text{g}/\text{ml}$  LPS at 37°C, in a 5% CO<sub>2</sub>-containing atmosphere. Enlarged cells, referred to as activated cells, could be detected after 24 h of culture, while proliferation was apparent after 48 h; this only occurred if LPS was present in the culture medium. Cells from 2–5-d-old cultures were used for electrophysiological recordings.

#### *Electrophysiological Experiments*

Just before recording (at 22–24°C), the cells were diluted in the extracellular bathing medium and allowed to settle for 5–10 min on the bottom of a 35-mm Petri dish on the stage of an IM-35 Zeiss inverted microscope (Carl Zeiss, Inc., Thornwood, NY). For ripped-off patch experiments, the dish was coated with polylysine (25  $\mu\text{g}/\text{ml}$ ; Sigma Chemical Co.) for 15 min, extensively washed, and allowed to dry for > 1 h before the cells were added. This procedure induced the cells to stick to the bottom of the dish enough so that isolated patches could be pulled off. Patch-clamp experiments (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) were carried out on enlarged LPS-stimulated B cells. Patch electrodes (5–10 M $\Omega$ ) were pulled from soft glass and coated with Crown sticky wax (SS White Ltd., Kingstone, UK). A 200–400- $\mu\text{m}$ -diam perfusion pipette was positioned near the recorded cell (at one to two pipette diameters) and connected to an eight-way tap to allow switching between different solutions. Monitoring the resistance of a patch electrode positioned in front of the outflow of the perfusion pipette during alternate application of Ringer and distilled water showed that this system could achieve a complete change of medium around the cell within 20 s. The perfusion flow was opened as soon as the whole-cell recording mode was established, and was continued throughout the whole experiment. The flow out of the chamber was produced by steady aspiration through a glass capillary placed on a cotton chunk to avoid discontinuous changes in the medium level. Since properties of the potassium current vary during the first minutes after breaking into the cell (Choquet et al., 1987), at least 10 min were allowed to elapse before application of any drug. The stability of K<sup>+</sup> current was assessed during this time by applying depolarizing pulses (500–1,500 ms in duration) every minute.

#### *Protocol for Drug Application*

K<sup>+</sup> channels in lymphocytes are closed at hyperpolarized potentials and opened by depolarization. To test the effect of 4-AP, K<sup>+</sup> currents were evoked every minute, unless stated otherwise, by 500–1,000-ms depolarizing pulses to +20 mV from a holding potential of –80 mV. These conditions do not induce cumulative inactivation (Choquet and Korn, 1988b). Control test pulses were delivered until the voltage-activated current reached a steady state. The drug to be tested was then applied for 3–5 min, and the cell was maintained at its resting potential (usually –80 mV). After this period, voltage jumps were resumed and the associated currents were labeled in successive order (i.e., 1st, 2nd, . . . 4-AP). The same protocol was followed when returning to the control Ringer solution.

### *Solutions Used for Patch-Clamp Recordings*

For whole-cell recordings, the bath solution was composed of the following: 140 mM NaCl, 4 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 mM glucose, and 10 mM HEPES buffer. The pH (7.2 in control experiments) was adjusted with NaOH to the indicated values. The recording pipette was filled with the following: 90 mM KCl, 50 mM KF, 4 mM NaCl, 4 mM MgCl<sub>2</sub>, 0.5 mM CaCl<sub>2</sub>, 5 mM EGTA (pCa = 8), and 10 mM HEPES buffer (pH 7.2 adjusted with KOH). The HEPES concentration of the pipette solution was raised to 20 mM in the experiments where the pH of the solution was adjusted to values different from 7.2 in order to provide a good buffering of the internal pH of the cell. In a few experiments the buffer concentration was increased by isosmotic replacement of KCl and KF with K-HEPES.

For single channel recordings in the outside-out configuration, the solutions were the same as above. For inside-out records, the pipette was filled with the bath solution and immersed in the low calcium solution referred to in the preceding paragraph as the pipette solution.

### *Data Acquisition and Analysis*

Data were acquired on a video recorder (JVC) through a modified pulse code modulator (Sony, Paris, France) and digitized at 0.5–40 kHz sampling rate through a Labmaster board, installed in an IBM-AT computer (IBM, Paris, France) and controlled by PClamp software (Axon Instruments, Inc., Foster City, CA), which was also used for data analysis.

### *Single Channel Analysis*

For inside- or outside-out recordings, depolarizing pulses from –80 to 0 mV, 1 s in duration, were also used. Data were acquired at 2.5 kHz to analyze channel openings, and at 40 kHz to measure the delay between the onset of the voltage jump and the first channel opening. Channel open time and amplitude were analyzed with Fetchan (Axon Instruments, Inc.) on traces from which the leak current was subtracted. The latter was assessed by averaging four to six sweeps lacking channel openings. Recordings with multiple channel openings were excluded from analysis.

Single K<sup>+</sup> channels exhibit bursts of openings, apparent under the form of bursting activities (see Results). We used mean burst durations in our analysis of channel behavior. A burst was defined as a series of openings separated by closed times briefer than a certain constant  $T$  (Magleby and Pallotta, 1983). The definition of  $T$  that minimizes the error made on burst length estimation can be derived from the distribution histogram of closed times. For this channel, the fits of the closed time distributions led us to distinguish a fast ( $T \approx 0.8$  ms) followed by several slower components ( $>8$  ms) (data not shown). We thus considered all closing times greater than  $T = 4$  ms to be burst separators.

### *Dose-Response Curves*

The voltage-dependent K<sup>+</sup> conductance ( $G_k$ ) of each cell was calculated from the equation  $I_k/(V-E_k)$ , where  $I_k$  is the difference between the peak current, elicited by a large depolarizing step reaching the potential  $V$  (+20 or +40 mV), and the linear leak current. The latter was estimated by scaling the mean current elicited by three to five small depolarizing pulses below the threshold for activation of the outward current. Estimated from the Nernst equation,  $E_k$ , the K<sup>+</sup> reversal potential, was –89 mV under these conditions. The peak current elicited by depolarizing pulses reaching +20 mV was measured during perfusion of the recorded cell with solutions containing increasing concentrations of the blocker, each solution being perfused until the current had reached a steady-state amplitude (3–5 min). After subtraction of the leak current, this value was normalized with respect to the control currents,  $I$ , recorded before

introduction of the blocker in the bath. Dose-response curves were established by averaging three to four separate experiments. Experimental points,  $I\%$ , were fitted by the equation  $1/[1 + (B/K_d)]$ , which expresses a one to one molecule interaction between the channel and the blocker, where  $B$  is the concentration of the blocker and  $K_d$  the equilibrium dissociation constant.  $K_d$  was derived from the linear regression of  $1/I\%$  as a function of  $B$ .

#### *Exponential Fits of Whole-Cell Currents*

The decaying phase of the voltage-activated K<sup>+</sup> current usually consisted of ~400 digitized points and was fitted by a single or double exponential function using a nonlinear least-squares fit. The correlation coefficient was used to estimate the goodness of the fit, and was >0.9 in all cases presented in this study. A double exponential fit was used when it led to a significant decrease (>1.5 times) in the  $\chi^2$  over a single exponential fit. Using a method based on the combined use of the Laplace transform and of Padé approximants (Yeremian and Claverie, 1987), we confirmed the mono- or bi-exponential nature of the experimental curves.

#### *Computer Simulations of Channel Behavior*

Channel behavior was modeled using the spreadsheet program 123 (Lotus Corporation, Paris, France). The number of channels in each kinetic state  $C_j$  at the instant  $t + dt$  was calculated from:

$$C_j(t + dt) = C_j(t) + dt \left[ \sum_{i=1}^n K_{C_i C_j} C_i(t) - \sum_{i=1}^n K_{C_j C_i} C_j(t) \right]$$

where  $K_{C_i C_j}$  is the rate constant from state  $C_i$  to state  $C_j$ , and  $n$  is the total number of kinetic states. The initial values  $C_i(0)$  were determined as described in the text.  $dt$  was set to 1 ms.

## RESULTS

### *Time and Depolarization Dependences of the Block of Whole-Cell K<sup>+</sup> Currents by 4-AP*

Fig. 1 illustrates a typical effect of 4-AP (500  $\mu$ M) on voltage-activated K<sup>+</sup> currents. Current block developed slowly as the cell was regularly depolarized by short (15 ms) duration pulses. The current elicited by the first pulse after addition of 4-AP was only marginally reduced (trace labeled 1 in Fig. 1 A), and five successive pulses were required before a steady-state level of block was reached. Similarly, after removal of 4-AP from the bath, recovery of the current only began after application of a depolarizing pulse. Several pulses were necessary before the current recovered to control levels (Fig. 1 B). In this and similar experiments, we allowed a 3-min lag period between 4-AP addition or removal and the beginning of the test pulses (Fig. 1 C). These slow kinetics were not due to a slow diffusion of 4-AP in the bath, since the medium around the cell changed completely in <20 s (see Methods).

These data suggest that membrane depolarization is required for the block and unblock to occur. This dependence on voltage might result if the binding of 4-AP to the channel depends directly on transmembrane voltage or if it depends on the open or closed state of the channel. We first tested the effect of voltage on 4-AP binding by using depolarizing pulses of different amplitudes above the threshold of channel activation. We measured 4-AP action at the steady-state level of block to eliminate the possible influence of a voltage dependence of channel open probability. In three

cases, the percentage of blockade at  $-30$  mV was different from that at  $+20$  mV by only 3.6% (SD = 4.7%) and  $-5\%$  (SD = 14%), for a 100- and a 500- $\mu\text{M}$  dose of 4-AP, respectively. In contrast, when the duration of the pulses was increased to values  $> 500$  ms, the steady-state level in the percentage of blocked channels was reached after a single depolarizing pulse was delivered. In seven experiments, the amplitudes of the currents elicited by the second and third pulses were only different by 6% (SD = 5%,  $n = 7$ ). Thus, in the range  $-30$  to  $+20$  mV, 4-AP blocks a fraction of the current which depends on the time spent at depolarized levels, rather than on the transmembrane voltage itself.

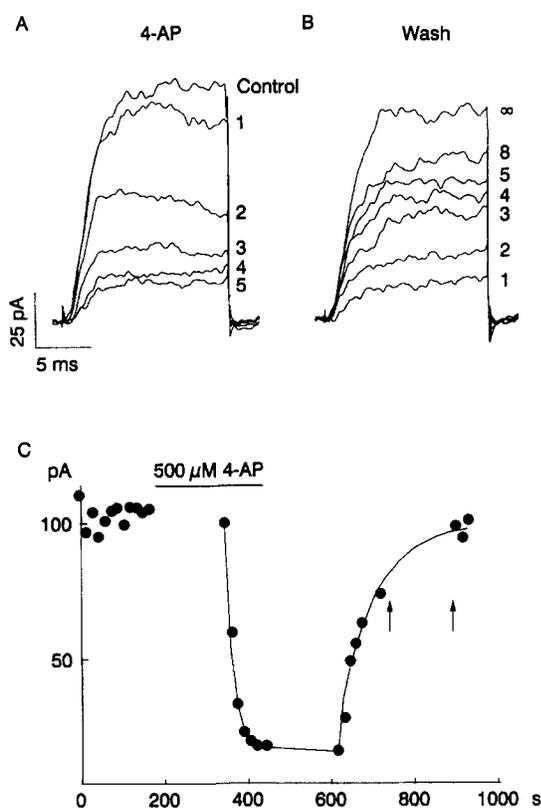


FIGURE 1. Block and unblock of voltage-gated  $\text{K}^+$  channels by 4-AP develop slowly. Whole-cell voltage-activated  $\text{K}^+$  currents elicited in a B lymphocyte by depolarizing pulses reaching  $+20$  mV, from a holding potential of  $-80$  mV. (A) The cell was perfused for 3 min with 500  $\mu\text{M}$  4-AP before successive voltage jumps were applied every 15 s (currents labeled in temporal sequence, the control one being recorded in the absence of the drug). The current decreased progressively. In the absence of 4-AP, voltage jumps at this frequency did not lead to cumulative inactivation. (B) Slow recovery of currents recorded during wash-out of 4-AP. The voltage jumps were applied as in A. (C) Plot of the amplitude of  $\text{K}^+$  current (ordinate) versus time (abscissa) during the same experiment. The onset and recovery from block are fitted by exponential functions,

having time constants of 19 and 90 s, respectively. The cell was subjected to long depolarizations (500 ms) in the time interval between arrows.

#### Concentration Dependence of 4-AP Action

The mechanism of 4-AP action was further investigated by analyzing the concentration dependence and detailed kinetics of the current block using long pulses of 500–1,000 ms. Fig. 2 illustrates an experiment where four concentrations of 4-AP were applied. For each of them, three current sweeps were recorded; the control, the first, and the second ones elicited by voltage pulses delivered, respectively, just before, and 4 and 5 min after introducing the drug to the bath.

For a 100- $\mu$ M dose of 4-AP, which approximates the  $K_d$  of channel block (Amigorena et al., 1990), we observed (Fig. 2A) that, although the peak amplitude of the current elicited by the first depolarizing pulse after 4-AP addition ( $I_{4\text{-AP}}$ ) was comparable to that recorded in absence of the drug ( $I_{\text{control}}/I_{4\text{-AP}} = 0.95$ , SD = 0.1,  $n = 6$ ; see also Fig. 3), its rate of decay was faster than the control (Table I). In contrast, the current elicited 1 min later by a second pulse in the presence of 4-AP was reduced in amplitude to about half its initial value ( $I_{\text{control}}/I_{4\text{-AP}} = 0.55$ , SD = 0.1,  $n = 6$ ; see also Fig. 3), while its rate of decay was slower than that of both the control and the first pulse (Table I).

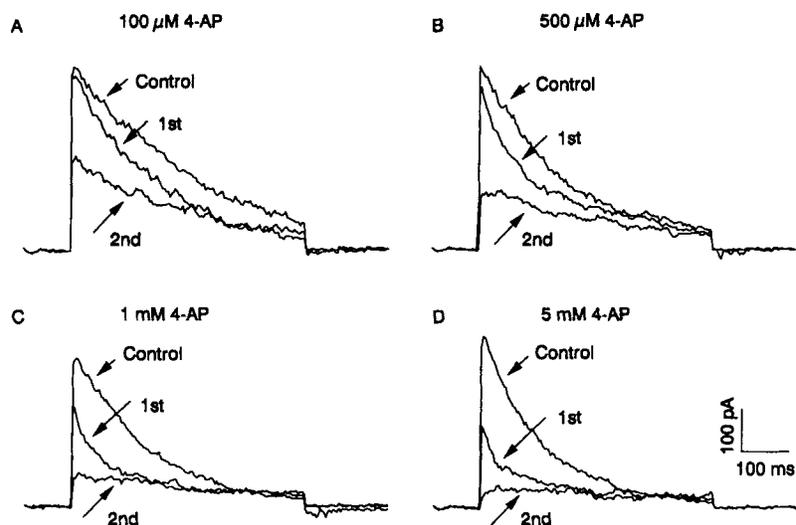


FIGURE 2. Evidence that the blocking action of 4-AP requires cell depolarization. Families of whole-cell  $K^+$  currents elicited by depolarizations to +20 mV. A depolarizing pulse was first applied in absence of the drug (control) and 4-AP, at the indicated concentrations, was then perfused for 4–5 min, during which the cell was continuously held at the holding potential (–80 mV). A depolarizing pulse was then applied and, as indicated, was followed 1 min later by a second one (1st and 2nd, respectively). Note the differences between the amplitudes and kinetics of the corresponding currents. Same cell from A to D recorded for 53 min; the drug was washed out by perfusing with control Ringer for several minutes, between each 4-AP application.

The recordings obtained at higher doses of 4-AP are shown in Fig. 2, B–D. At all doses of 4-AP, the first  $K^+$  current elicited 4–5 min after drug addition had a faster decay than the control, and the rate of decay increased as the concentration of the drug was raised (Table I). A decrease in the peak of the first current, as compared with control, was apparent at 500  $\mu$ M, and was more pronounced at higher doses. However, block of the peak current was much more apparent when the second depolarizing pulse was applied. In addition, the current elicited by the second pulse decayed more slowly, as 4-AP concentration was increased (Table I). As stated before, additional pulses did not markedly modify the recordings. The difference between

TABLE I  
*Time Constants of the Exponential Fits to the Decaying Phase of the K<sup>+</sup> Currents  
 Obtained in Control Conditions and in the Presence of 4-AP*

	Dose of 4-AP			
	0.1 mM (n = 8)	0.5 mM (n = 6)	1 mM (n = 12)	5 mM (n = 3)
Control (ms)	169 ± 47	133 ± 32	120 ± 17	163 ± 48
First 4-AP				
T <sub>1</sub> (ms)	42 ± 12	36 ± 12	34 ± 20	20 ± 2.5
T <sub>2</sub> (ms)	245 ± 75	275 ± 164	399 ± 215	368 ± 264
A <sub>1</sub> /A <sub>2</sub>	1.07 ± 0.56	1.9 ± 1.1	2.7 ± 1.5	5.0 ± 2.8
Second 4-AP				
T <sub>1</sub> (ms)	185 ± 30	190 ± 60	394 ± 123	ND

The inactivation phase of currents recorded in the absence of the drug (control) or elicited by the second pulse in the presence of 4-AP (second 4-AP) were fitted by a single exponential function, whereas the current produced by the first pulse delivered after addition of the blocker was fitted with the sum of two exponential functions, with the indicated time constants (T<sub>1</sub> and T<sub>2</sub>). A<sub>1</sub>/A<sub>2</sub> is the ratio of the amplitudes of the associated exponential functions. Currents were produced by 500–1,000-ms voltage steps from a holding potential of –80 to +20 mV. In this and the following tables, values are given ± SD. ND, not determined.

the amount of block of the current elicited by the first depolarizing step and that of subsequent sweeps, at the various doses of 4-AP, is illustrated in Fig. 3.

The channel block could not be relieved by cessation of the pulsing. We waited up to 10 min after a steady-state block was reached in the presence of 4-AP without depolarizing the cell. When pulses were resumed, the current was still decreased, in a manner similar to the current observed before this lag period (data not shown). Thus, while the cell needs to be depolarized for block to occur, the block is not relieved when the cell is held at hyperpolarized potentials.

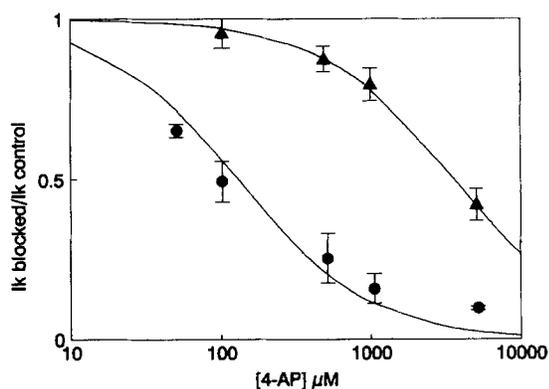


FIGURE 3. Dose-response curves of 4-AP action. Percentage of K<sup>+</sup> current remaining in the presence of increasing concentrations of 4-AP, normalized with respect to control currents recorded in absence of drug. ▲, Peak amplitudes of the currents produced by the first depolarizing pulse, after addition of 4-AP. ●, Peak amplitudes at steady-state block after at least two pulses. Experimental points are fitted to the function 1/(1 +

[4-AP]/K<sub>d</sub>), which assumes a one to one molecular interaction between the channel and the blocker (K<sub>d</sub>'s of 3.12 and 0.130 mM, respectively). Each point is a mean of three to four experiments. Error bars are ± SD.

*Time and Depolarization Dependences of the Recovery from the Block by 4-AP*

After removing 4-AP, the recovery of K<sup>+</sup> current amplitude occurred slowly when short (15 ms) pulses were applied. However, this unblocking process was considerably speeded up when long depolarizing pulses were used. In the presence of 100  $\mu$ M 4-AP (Fig. 4 A) and in steady-state conditions, the K<sup>+</sup> current was reduced to half its control value. Surprisingly, the K<sup>+</sup> current elicited by the first pulse after an intensive wash-out period of at least 4 min had only slightly recovered its amplitude (9% increase, over the current recorded during the steady-state block; SD = 6%,  $n = 3$ ). In contrast, the current produced by the second pulse was fully restored (98% of the peak amplitude of control currents; SD = 0.4%,  $n = 3$ ).

A similar experiment carried out on the same cell incubated with 5 mM 4-AP before wash-out is illustrated by Fig. 4 B. The current elicited by the first voltage step

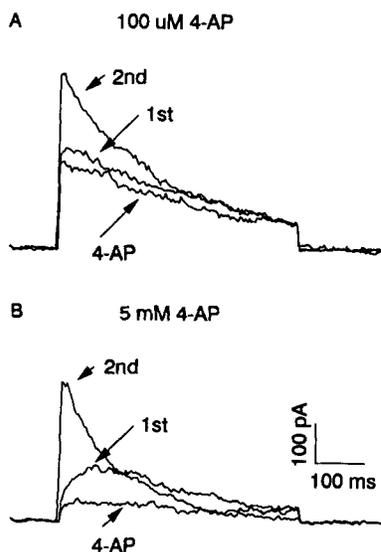


FIGURE 4. Effect of cell depolarization on the unblocking of K<sup>+</sup> channels. Whole-cell K<sup>+</sup> currents elicited by depolarizing the cell to +20 mV. A steady-state response was recorded in the presence of 100  $\mu$ M (A) and 5 mM 4-AP (B). The drug was then washed out for 4–5 min, during which the cell was continuously held at the holding potential (–80 mV), and currents were then regularly elicited at 1-min intervals. Note that, with both doses, the first pulse still reveals a marked block, while the second pulse elicits a fully recovered current. Same cell for both A and B; the drug was washed out by perfusing with control Ringer during several minutes between each 4-AP concentration.

after wash-out had partially recovered its amplitude; however, both the rising and the decaying phases of the current were slower than those of currents recorded in control conditions or in the presence of 4-AP (Table II). Similar results were observed in other cells preincubated with 1 mM 4-AP ( $n = 6$ ) or 5 mM 4-AP ( $n = 3$ ). This slow time course may result from the progressive unblocking of channels during the application of the depolarizing step. As in the case of preincubation with 100  $\mu$ M 4-AP, a nearly complete relief of the block was achieved at the time of the second pulse after wash (Table III).

These data suggest that channel unblocking requires depolarization and cannot occur while the cell is hyperpolarized; however, the dependence on voltage of channel unblocking is due to the trapping of 4-AP in closed channels rather than to the effect of the membrane potential itself on the binding of 4-AP. The block of the channel requires depolarization, and once block is established the unblocking process

TABLE II  
*Time Constants of the Exponential Fits to the Slow Rising ( $T_r$ ) and Decaying ( $T_d$ )  
 Phases of the First K Currents Elicited after Wash-out of 4-AP*

	Dose of 4-AP			
	0.1 mM (n = 4)	0.5 mM (n = 4)	1 mM (n = 6)	5 mM (n = 3)
Control (ms)	202 ± 39	176 ± 35	171 ± 64	162 ± 48
$T_r$ (ms)	ND	ND	26 ± 15	39 ± 9
$T_d$ (ms)	290 ± 74	363 ± 228	298 ± 94	384 ± 130

Control values are for inactivation time constants of currents recorded before addition of the blocker. Some of the cells are the same as those in Table I.

can only occur if the cell is depolarized. Furthermore, the potency of 4-AP does not vary when depolarizing steps reaching  $-30$  or  $+20$  mV are used.

#### *Kinetic Analysis of Whole-Cell Currents*

As previously reported (Choquet and Korn, 1988b), the inactivation phase of the voltage-dependent  $K^+$  currents could be fitted in control conditions by a single exponential function. In contrast, the activation process displays a more complex behavior, since a fourth- to sixth-order power function is required for adequate fit (see also Cahalan, Chandy, DeCoursey, and Gupta, 1985).

There were marked differences in kinetics among the currents elicited by the first depolarizing pulse after the addition of the drug, that evoked by the second voltage step, and the first current after removal of 4-AP.

(a) The decay phase of the  $K^+$  current elicited by the first depolarizing pulse after addition of 4-AP could be fitted by the sum of two exponential functions in most of the cases ( $n = 29$  of 34; Fig. 5). As the drug concentration was increased, the first component became faster while the second one became slower, and the relative amplitude of the first versus the second component increased. These facts are reflected by the mean values of the time constants and by the amplitudes of the exponential functions given in Table I. The slow decline at high doses of 4-AP leads to crossovers between the currents recorded in the presence of 4-AP and in the

TABLE III  
*Relief of Channel Block by Depolarizing Pulses after Wash-out of 4-AP*

	Dose of 4-AP			
	0.1 mM (n = 3)	0.5 mM (n = 3)	1 mM (n = 5)	5 mM (n = 3)
First pulse (%)	9 ± 6	7 ± 4	22 ± 2	22 ± 4
Second pulse (%)	98 ± 0.4	82 ± 7	89 ± 6	90 ± 6

The percentage of channels unblocked after wash-out of 4-AP was derived from the equation:  $(I_{\text{pulse}} - I_{\text{blocked}}) / (I_{\text{control}} - I_{\text{blocked}})$ , where  $I_{\text{pulse}}$  is the amplitude of the  $K^+$  current elicited by the first or second pulses, respectively, after wash-out of the drug and  $I_{\text{control}}$  and  $I_{\text{blocked}}$  are the peak amplitudes of the current in the absence and in the presence (at the indicated doses) of the blocker, respectively, under steady-state conditions. Only experiments where the duration of the pulse was  $>500$  ms are used. Note that whereas nearly all the channels were still blocked during the first depolarizing pulse, recovery was complete during the second one.

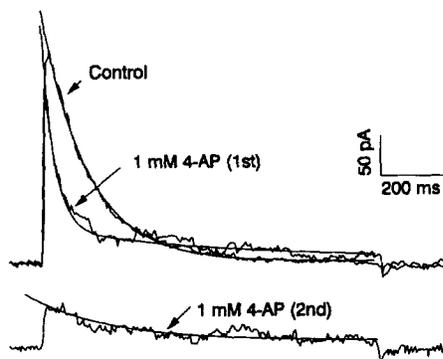


FIGURE 5. Exponential decay of control and 4-AP affected currents. Whole-cell outward currents elicited by 100-mV depolarizing pulses, in control conditions and in the presence of 1 mM 4-AP. After the addition of 4-AP to the bath, the cell was continuously held at the holding potential (-80 mV) for 3 min. Traces labeled 1st and 2nd were then triggered at 1-min intervals. Single (control, 2nd) and double (1st) exponential fits to the decaying phase of the currents are superimposed on the experimental record. Time constants are: control, 133 ms ( $R = 0.998$ ); 1 mM 4-AP (1st), 36 ms and 525 ms ( $R = 0.98$ ); 1 mM 4-AP (2nd), 238 ms ( $R = 0.91$ ).

control medium (Figs. 2 and 5). This suggests that blocked channels do not inactivate; a similar effect of 4-AP has been observed on  $I_A$  K currents (Thompson, 1982).

(b) Inactivation of the current elicited by the second pulse after addition of 4-AP was always slower than the inactivation of the control current, a phenomenon that was more apparent as the concentration was increased. Inactivation could be fitted with a single exponential in most cases, except after exposure to 5 mM 4-AP, since at this dose the remaining current was small. The presence of slow phases of decay in all currents recorded in the presence of 4-AP suggests that, after  $K^+$  channels are unblocked during depolarizing pulses, the newly unblocked channels may inactivate.

(c) After wash-out of 4-AP (Fig. 6), current traces could be fitted with the sum of two exponential functions: one for the slow phase of activation and the other for the decay (the mean values for the associated time constants are listed in Table II). The

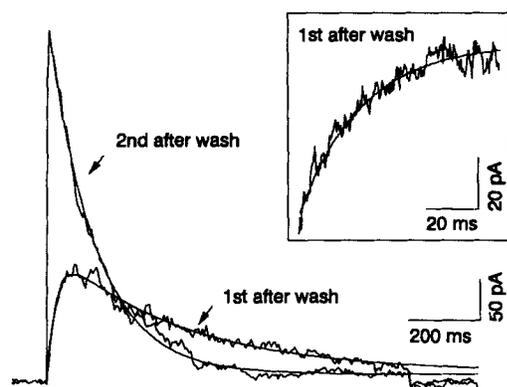


FIGURE 6. Exponential fits of currents during the recovery from block by 5 mM 4-AP.  $K^+$  currents elicited by the first and the second pulse 3 and 4 min after the beginning of the wash-out. The traces were fit by the sum of two exponential functions, one for the rising ( $t_{rise}$ ) and the other for the decaying phase ( $t_{decay}$ ). Time constants are  $t_{rise} = 25$  ms and  $t_{decay} = 350$  ms ( $R = 0.986$ ) for the first after wash, and  $t_{rise} = 2.6$  ms and  $t_{decay} = 125$  ms ( $R = 0.997$ ) for the second current after wash. The inset shows an expanded view of the rising phase of the first current recorded after wash out. The cell was preincubated for 5 min with 4-AP before wash-out.

rising phase of this wash-out current appeared to follow a first-order process (Fig. 6, *inset*), in contrast to the fourth- to sixth-order kinetics during the rising phase of control currents (not shown). Since, as stated above, these kinetics indicate a slow unblocking process of the channels, our data could indicate that the unbinding of 4-AP occurs within a single step in the series of transitions that are thought to occur between the closed and the open states of the channel.

#### *Single Channel Analysis of K<sup>+</sup> Channel Block by 4-AP*

To examine the site of 4-AP action, experiments were performed at the single channel level. Fig. 7 is typical of the recordings obtained in the outside-out mode. In control conditions, depolarizing pulses applied every minute activated unitary currents carried by K<sup>+</sup> (which is the only ion with a positive driving force at the 0 mV recording potential). In these illustrated recordings, three equidistant levels of

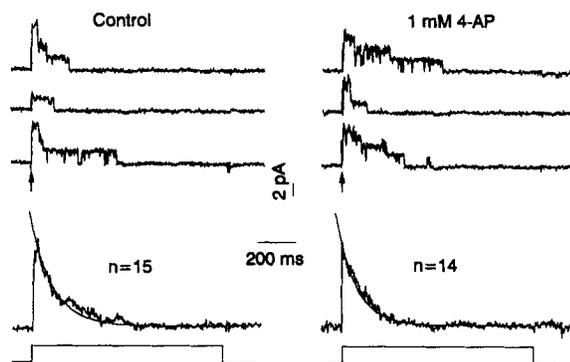


FIGURE 7. 4-AP applied on the extracellular side of an outside-out patch does not affect the activity of the K<sup>+</sup> channel. Single channel activity during depolarizing pulses from -80 to 0 mV, 1 s in duration and given every 30 s. 15 and 14 sweeps, respectively, recorded in control condition (left-hand panel) and in the presence of 1 mM 4-AP (right-hand panel). Representative examples of

channel activity in both conditions (upper part) and averages of the sweeps (lower part). The latter are fit by single exponential curves (superimposed) having time constants of 112 ms ( $R = 0.98$ ) and 100 ms ( $R = 0.99$ ) in control and 4-AP conditions, respectively. Vertical scale bar: 1 pA. Data were filtered at 500 Hz and sampled at 2.5 kHz in this and the following figure. The arrow indicates the start of the depolarizing pulse.

openings are apparent; they indicate the presence of at least three identical channels in the patch. Channel activities were detected immediately after the voltage jump in most of the cases, and they decreased within a few hundred milliseconds. No openings were usually noticed toward the end of the depolarizing pulse. The openings were interrupted by brief closures which were not taken into account for calculation of open times (see Methods). Several arguments indicate that these channels underlie the voltage-dependent K<sup>+</sup> currents recorded in the whole-cell mode. (a) As in the latter, it was necessary to hold the patch at negative potentials for tens of seconds between voltage jumps in order to allow the channels to recover from inactivation. (b) Current traces obtained by averaging several unitary sweeps (Fig. 7) displayed characteristics similar to voltage-activated currents recorded in whole-cell mode. Namely, there was a fast activation followed by a slow inactivation of the current. The latter phase was adequately fitted by a single exponential, having a time

constant that was of the same order of magnitude, although faster, than those used for control whole-cell currents.

Addition of 1 mM 4-AP to the extracellular side of the same patch did not produce detectable changes in the pattern of channel activity. As seen in Fig. 7, the same number of channels were active in the patch. The amplitude of the unitary currents, as well as the mean burst time, were not affected significantly by 4-AP (mean amplitude and burst duration were 1.0 pA; SD = 0.11 pA and 77.1 ms; SD = 61.4 ms,  $n = 36$  in control conditions and 1.01 pA; SD = 0.12 pA and 71.4 ms; SD = 96.7 ms,  $n = 32$  in the presence of 1 mM 4-AP). Finally, the current traces obtained by averaging individual sweeps in the two experimental conditions (Fig. 7) displayed similar decay times (i.e., 112 and 100 ms in the control and in the presence of 4-AP, respectively). Thus, in lymphocytes 4-AP does not seem to block voltage-activated K<sup>+</sup> channels when applied at the external side of outside-out patches. Identical results were found in five separate experiments.

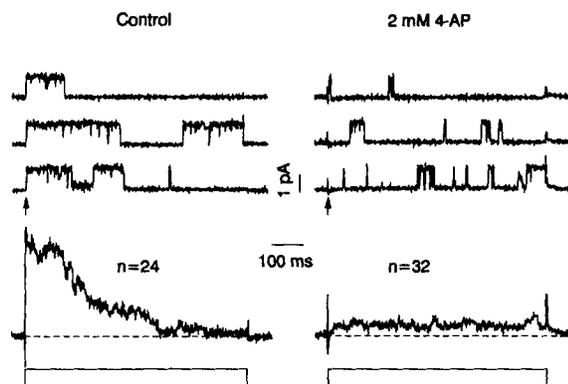


FIGURE 8. The effect of 4-AP applied on the intracellular side of an inside-out patch on the activity of the K<sup>+</sup> channel. Single channel activity elicited by depolarizing pulses from -80 to 0 mV, 700 ms in duration and given every 30 s. 24 and 32 sweeps recorded in control conditions and in the presence of 2 mM 4-AP. Typical traces (upper part) and averages (lower traces) are shown. Note the net decrease in the

duration of bursts in the presence of the drug. The pipette solution, facing the external part of the membrane, was standard Ringer's, while the bath solution, facing the internal face, was of the intracellular type. Vertical scale bar: 1 pA.

To apply 4-AP on the internal face of the membrane, we performed the same experiments as above in the inside-out configuration ( $n = 3$  experiments). As illustrated in Fig. 8, the control channel activity displayed an overall behavior similar to that recorded in the outside-out mode with similar single channel conductances and mean burst durations, although the latter were slightly longer (77.1 ms, SD = 61.4 ms,  $n = 36$  in outside-out, versus 99 ms, SD = 104 ms,  $n = 46$  in inside-out). Addition of 2 mM 4-AP to the intracellular side of the membrane had no effect on single channel amplitude (1.17 pA, SD = 0.16 pA,  $n = 46$  in the control, versus 1.06 pA, SD = 0.08 pA,  $n = 63$  in the presence of 2 mM 4-AP). However, 4-AP had two effects on channel activity. First, it reduced the channel open time. In the illustrated experiment, the mean burst length in the presence of 4-AP was only 28% of the control value (mean control burst duration: 99.0 ms, SD = 104 ms,  $n = 46$ ; over 28.3 ms, SD = 24 ms,  $n = 63$  in the presence of 2 mM 4-AP,  $P < 10^{-6}$  using a paired Student's  $t$  test), with no detectable effect on channel flickering (closed

times < 4 ms; see Methods). Second, there was a large increase in the delay between the onset of the voltage jump and the first channel opening, which increased from 2 ms (SD = 0.72 ms,  $n = 29$ ) to 152 ms (SD = 176 ms,  $n = 25$ ,  $P < 5 \times 10^{-4}$  using a paired Student's  $t$  test). Since these recordings were performed at a steady-state level of channel block, this delay probably reflects the time needed for the channels to unblock before they can open. Finally, the probability of channel opening remained roughly constant during voltage steps applied in the presence of 4-AP. This effect is apparent on the averaged sweep of Fig. 8 where no inactivation can be detected. This result is reminiscent of the slower inactivation of whole-cell  $K^+$  currents in the presence of 4-AP (Table I).

#### Sensitivity of 4-AP's Potency to pH

These results show that 4-AP blocks  $K^+$  channels from the intracellular side of the membrane. Since whole-cell currents can be blocked by extracellular 4-AP, we asked how the drug crosses the membrane to reach its site of action.

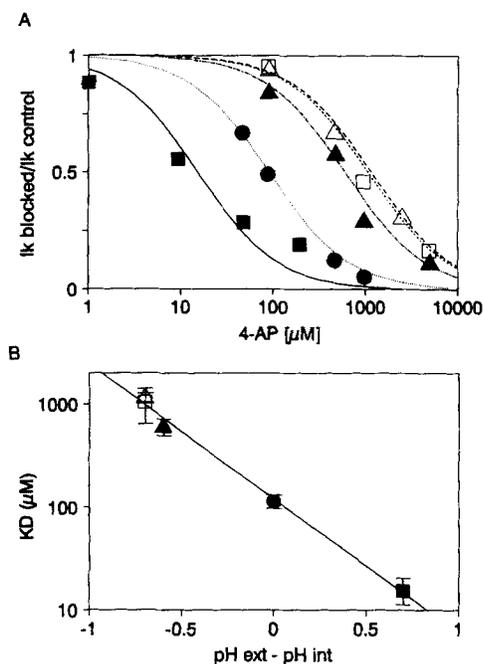


FIGURE 9. pH dependence of 4-AP potency. (A) Dose-response curves of whole-cell  $K^+$  current block by 4-AP in various internal and external pH solutions. SD omitted for clarity. (B) Plot of the mean dose of 4-AP inducing 50% inhibition of  $K^+$  currents ( $K_d$ ) as a function of the difference between the external and the internal pH. Experiments in which at least three different 4-AP concentrations were applied to a single cell were used to calculate the dose for 50% inhibition (see Methods). For each pH condition,  $K_d$  is the mean from three to four experiments. SD are indicated by the bars. The regression line drawn to the experimental points has a slope of  $-1.2$  ( $R = 0.994$ ). Symbols refer to the following conditions ( $pH_{\text{external}}$ ,  $pH_{\text{internal}}$ ): ■, (7.9, 7.2); ●, (7.2, 7.2); ▲, (7.7, 6.5); □, (7.2, 7.9); △, (6.5, 7.2).

4-AP is a weak base with a  $pK$  of 9.11 at 25°C. Thus, at physiological pH (7.2), ~99% of this drug is in the ionized form. We thus attempted to determine the contribution of the ionized and nonionized forms of 4-AP to the channel block via the influence of both internal and external pH. In the limited pH range that we used (6.5–7.9), the kinetics and voltage dependence of the control  $K^+$  currents were similar to those recorded in standard pH conditions (7.2 on both sides of the membrane), in agreement with other results (Deutsch and Lee, 1989).

Fig. 9A shows the dose-dependent inhibition of whole-cell, voltage-activated  $K^+$  currents in solutions of varying pH. 4-AP is less effective as the external solution is

made more acidic or as the internal solution is made more alkaline, a result that depends on both intra- and extracellular pH and which has also been observed recently in rabbit Schwann cells (Howe and Ritchie, 1991). The effectiveness of 4-AP does not depend primarily on either one of these values but rather on their algebraic difference. Comparable dose-response curves were obtained when the values for external and internal pH were 7.2 and 7.9 or 6.5 and 7.2, both cases having an identical  $\Delta\text{pH}$  of 0.7 (Fig. 9A). It was possible that addition of high concentrations of 4-AP to the extracellular medium changed the intracellular pH. In three experiments we raised the buffer concentration by isosmotic replacement of intracellular anions with HEPES and found a  $K_d$  (1.33 mM for a  $\Delta\text{pH}$  of 0.7) similar to that obtained with the HEPES concentration (20 mM) used in most experiments.

The  $K_d$  of the channel block derived from the curves in Fig. 9A indicated a linear relationship between the  $\text{p}K_d$  and external pH – internal pH, with a slope of 1.2 (Fig. 9B). These findings allow us to propose a model (Fig. 10) accounting for the relation

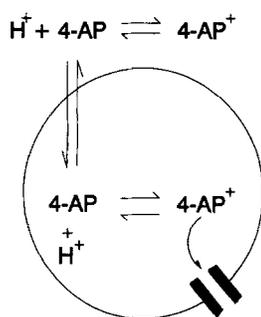


FIGURE 10. Model of the mechanism postulated to account for the action of 4-AP. As indicated, our results suggest that (a) 4-AP enters the cell in its nonionized form (4-AP) and redissociates into its ionized form (4-AP<sup>+</sup>) and (b) K<sup>+</sup> channels are blocked by the latter at their intracellular side (see text for further explanation).

between 4-AP potency and pH. We assume: (a) only the nonionized form (4-AP<sub>nonionized</sub>) can cross the plasma membrane, and that this form is in equilibrium across it. (b)  $[4\text{-AP}_{\text{nonionized}}] \ll [4\text{-AP}_{\text{ionized}}]$ , since the pH of the experiments was far from the  $\text{p}K$  of 4-AP ionization. These assumptions lead to the relation:

$$[4\text{-AP}_{\text{ionized}}]_{\text{internal}} = ([\text{H}^+]_{\text{internal}}/[\text{H}^+]_{\text{external}}) \cdot C$$

where  $C$  is the total concentration of 4-AP. This equation can be written:

$$\text{p}K_{\text{channelblock}} = C' - (\text{pH}_{\text{internal}} - \text{pH}_{\text{external}})$$

Thus, with this relationship, the potency of 4-AP depends linearly on the difference between internal and external pH (at least for  $\text{pH} \ll \text{p}K_{4\text{-AP}}$ ), with a slope of 1, which is in close accord with the experimental value of 1.2 mentioned above.

The question remains whether the ionized or nonionized form of 4-AP blocks the channel. If, as we assume, the nonionized form is at equilibrium across the membrane, then varying the internal pH modifies the total internal 4-AP concentration by acting solely on  $[4\text{-AP}_{\text{ionized}}]_{\text{internal}}$ . Since the block of the channels is sensitive to internal pH, it is likely that 4-AP<sub>ionized</sub> does block the channels, but the effectiveness of 4-AP<sub>nonionized</sub> was not ruled out by our experiments. However, 3-aminopyridine (3-AP) and 3,4-diaminopyridine (di-AP), which are closely related to 4-AP, are respectively neutral and ionized at physiological pH. While 3-AP is a poor blocker of K<sup>+</sup> channels,

whether applied outside or inside the cells, di-AP has a potency similar to 4-AP (Thompson, 1982). These data could suggest that 4-AP<sub>nonionized</sub> does not affect K<sup>+</sup> currents.

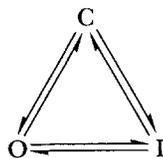
#### DISCUSSION

Two arguments suggest that the binding of 4-AP depends on the state of the channel, which varies with transmembrane voltage, rather than on membrane potential itself. First, there was no significant difference in the potency of 4-AP at the steady-state level of block between depolarizing steps to  $-30$  and  $+20$  mV. Second, channel block as well as unblock occur only at depolarized potentials. The last observation shows that 4-AP does not unbind from the channels at hyperpolarized potentials, as would be the case if 4-AP displayed voltage-dependent binding to the channel.

Single channel experiments in isolated patches demonstrate that 4-AP only blocks K<sup>+</sup> channels from the internal side of the membrane. The pH dependence of 4-AP action suggests that, in whole cells, the drug crosses the membrane in its nonionized form and blocks the channels from the inside once reionized.

#### *Kinetic Modeling of 4-AP Action*

On the basis of experimental data and computer simulations, it is proposed here that both the blocking and unblocking of channels by the drug first require opening of channels and that 4-AP binds to both open and inactivated channels. We shall consider the simplest model for channel behavior in control conditions with three states, the closed (C), open (O), and inactivated (I) ones:



This model is an oversimplification since the kinetics of channel activation indicate the existence of at least four gates for channel opening, and since this voltage-dependent K<sup>+</sup> channel displays cumulative inactivation there must be at least two inactivated states (Hodgkin and Huxley, 1952; Cahalan et al., 1985; Lee and Deutsch, 1990). However, this approach could be justified since (a) channel activation is more than an order of magnitude faster than channel block or inactivation, and (b) in our control conditions the rate of inactivation of the channel is well described by a single exponential.

#### *4-AP Cannot Enter or Leave Closed Channels*

Experimental evidence that 4-AP poorly binds to closed channels is shown by dose-response curves as in Fig. 3. However, above 1 mM, currents elicited by the first pulse after drug addition display a certain amount of block. If we assume that (a)

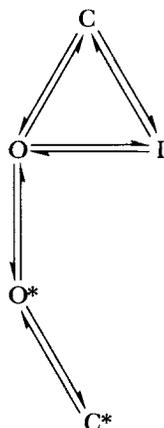
channel activation follows a Hodgkin-Huxley type of kinetics,  $C \cdot [1 - e^{-(t/T)}]^4$ , with a time constant  $T$  of 1 ms, and (b) 4-AP binds to open channels with a rate of  $20 \text{ s}^{-1}$  (which corresponds to a 5-mM dose), then, based on computer simulations, one would expect a 25% reduction in peak current amplitude due to a fast block of the channels during the activation process. This value represents approximately half of the 58% decrease observed experimentally. Thus, either there is some binding of 4-AP to closed channels (with, in this case, a  $K_d$  greater than 10 mM, as derived from Fig. 3), or 4-AP blocks the channels during infrequent openings at hyperpolarized potential. Testing this second hypothesis would require investigating the probability of opening of the channels at these voltages.

Despite the very low affinity of 4-AP for closed channels, different sets of data show that 4-AP can remain trapped in closed channels at hyperpolarized potentials. The most direct evidence is that, after wash-out, channels unblock only if the cell is depolarized. Along this line, there is no recovery from block between depolarizing pulses in the presence of 4-AP. Thus channels can close with bound 4-AP, and this process prevents unbinding. The notion that molecules of blockers can be trapped in closed channels was first proposed by Armstrong (1971), who described the block of K<sup>+</sup> channels of squid axon by quaternary ammonium compounds, and has been generalized for other channels (reviewed in Hille [1984], such as calcium ions trapped in K<sup>+</sup> channels of lymphocytes [Grissmer and Cahalan, 1989a]).

#### *4-AP Blocks Open Channels*

Converging arguments demonstrate the importance of the open state during the process studied here. The accelerated rate of decay of the current in the presence of the drug and the reduction in channel open time both indicate that channels leave the open state faster in the presence of 4-AP than under control conditions. Also, the persistence of channel openings even at the end of long depolarizing pulses in the presence of 4-AP indicates that the drug does not increase the rate of transition to the inactivated state. This notion pertains both to direct observations in inside-out experiments, and to the slow kinetics of current inactivation in whole-cell recordings. As previously suggested (Thompson, 1982), open but blocked channels do not inactivate, or at least do so more slowly than unblocked channels, as further indicated by the crossovers between control current traces and those recorded either in the presence of 4-AP or after wash-out of 4-AP. Furthermore, our observations show that unblocking of the channels occurs during depolarizing pulses.

The rate constants for 4-AP binding to open channels and for channel unblocking can be obtained from the exponential fits of the experimental curves. Let us consider a model with one open but blocked state, O\*, from which 4-AP can bind and unbind at depolarized potentials, and one closed, blocked state, C\*, in which 4-AP is trapped at hyperpolarized potentials. The experimental data show that 4-AP cannot access closed channels; thus there is no transition from C to C\*, or from O\* to C. Moreover, we assume that channels only go from O\* to C\* upon cell repolarization, which implies a single blocked state at depolarized potentials.



The rate constants from open to blocked ( $K_{oo^*}$ ) and from blocked to open ( $K_{o^*o}$ ) are obtained from:

$$K_{oo^*} = (T_2 - T_i)(T_i - T_1)/(T_2 \cdot T_1 \cdot T_i)$$

$$K_{o^*o} = T_i/(T_1 \cdot T_2)$$

where  $T_i$  is the inactivation rate constant in control condition, and  $T_1$  and  $T_2$  are the two time constants of a double exponential fit to the first current elicited after addition of 4-AP. Moreover, if we assume that the slow activation phase in the first pulse after wash represents unblocking of channels, then  $K_{o^*o}$  is also the inverse of the time constant of this unblocking phase. The values of  $K_{o^*o}$  and  $K_{oo^*}$  derived from the experimental data are given in Table IV.

#### *Evidence That 4-AP Blocks Inactivated Channels*

In contrast to this simple model, we must postulate that 4-AP also binds to inactivated channels. If 4-AP can only bind to open channels, then only a small fraction of the channels would be blocked at the end of a depolarizing pulse (i.e., at the steady state

TABLE IV  
Rate Constants of Channel Block ( $K_{oo^*}$ ) and Unblock ( $K_{o^*o}$ ) in the Model with a Single Blocked State

	Dose of 4-AP			
	0.1 mM (n = 4)	0.5 mM (n = 4)	1 mM (n = 3)	5 mM (n = 3)
$K_{oo^*}$	6	10	15	24
$K_{o^*o}$	16	14	9	26
$K_{o^*o}$ wash	ND	ND	38	26

Rate constants were calculated from the mean time constants of the exponential fits (Tables I and II) to the currents recorded either in the presence of 4-AP ( $K_{oo^*}$ ,  $K_{o^*o}$ ) or during the first pulse after wash-out ( $K_{o^*o}$  wash) of the drug (see text for details).

for the current), most of them being inactivated. The lack of channel block in this case is due to the relatively slow transitions from the open to the blocked state. For example, computer simulations based on experimentally derived values for the rate constants at 1 mM 4-AP indicate that if  $K_{o*o} = 10 \text{ s}^{-1}$  and  $K_{oo*} = 15 \text{ s}^{-1}$ , then 4.1% of the channels are blocked at the steady state, a value becoming only 0.9% if  $K_{o*o} = 40 \text{ s}^{-1}$  and  $K_{oo*} = 15 \text{ s}^{-1}$ . By contrast, our experimental data indicated that most of the channels are blocked when the second pulse is delivered (84% of the channels are blocked at the beginning of the second pulse in the presence of 1 mM 4-AP).

The contribution of 4-AP binding to open channels during the tail current is certainly negligible, since block is less pronounced when short depolarizing pulses are used, i.e., when tail currents are maximum. Thus, a large proportion of the channels become blocked when they are in a nonconductive state. This may occur during the depolarizing pulse itself, or between the first and second pulses. The last hypothesis would imply that depolarizing the cell makes closed channels sensitive to 4-AP. Such would be the case if, for example, 4-AP needs to be "pushed" by the depolarization, in order to reach a site where it can bind to closed channels. Alternatively, if the block is completed during the first depolarizing pulse, then the only channel state candidate for block is the inactivated state. As shown for O\*, upon repolarization inactivated blocked channels (I\*) must proceed to a closed blocked state C\*, from which 4-AP cannot escape at hyperpolarized potentials.

#### *Evidence That Channel Unblocking Proceeds through Two Pathways*

We have direct access to the kinetics of unblocking of channels through the time course of the current trace recorded just after wash-out of 4-AP. If we assume that, for a high concentration of 4-AP, 100% of the channels are in a single blocked state, C\*, at rest, and that the kinetics from C\* to O\* are similar to those from C to O (that is, very fast), then the experimental record should follow the equation:

$$\text{number of open channels at } t = A[K_{O^*O}/(K_{OI} - K_{O^*O})][e^{(-tK_{O^*O})} - e^{(-tK_{OI})}]$$

where  $A$  is the total number of channels available for activation. That is, with this model, the time constant of the exponential function fit to the decaying part of the current should be equal to  $1/K_{OI}$ . Such is obviously not the case since, as shown in Fig. 6 and Table II, the time constants derived from experimental data are significantly slower (300 ms) than the control time constant of inactivation (180 ms). We must thus suppose that channels are in two different blocked states (C\* and C'\*) at rest, which upon depolarization evolve toward states from which 4-AP can unbind with two kinetics: (a) a fast one, which would account for the rise in the current elicited by the first pulse after wash; and (b) a slow one accounting for the current's slow decay.

#### *Modeling the Various Effects of 4-AP*

Combining the different hypotheses about the blocking kinetics, we obtained a model, chosen among several as the simplest, which accounts for most of the observed data: at depolarized potentials 4-AP can bind and unbind to open (O) or inactivated (I) channels. In the absence of direct access to the rate constants between

the inactivated and blocked inactivated ( $I^*$ ) channels, we arbitrarily chose them as equal to those between  $O$  and  $O^*$ .

Upon repolarization, inactivated channels proceed to the closed state  $C$ , while channels in  $I^*$  and  $O^*$ , respectively, proceed to two distinct closed blocked states,  $C^*$  and  $C'^*$ . As stated above, we can account for the shape of the currents elicited by the second pulse after addition of 4-AP and after wash-out by imposing different rate constants for the transitions from  $C'^*$  to  $O$  and from  $O^*$  to  $O$  (an eventual intermediate state between  $C'^*$  and  $O$  being arbitrarily suppressed since it is not essential to the model).

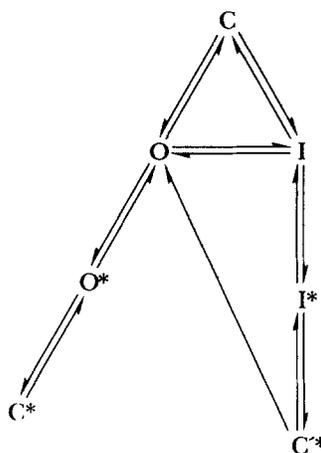


Fig. 11 shows the theoretical curves obtained with this model from the experiment presented in Fig. 5. The first current in the presence of 4-AP, as well as the amount of block of the second current and its slow decay rate, are well represented. However, there is a crossover between the decay phases of the first and second traces recorded in the presence of 4-AP, which was not observed experimentally. Fig. 12 shows the experimental curve from Fig. 6 (first after wash) superimposed with the theoretical curve corresponding to this model. In this case, at rest, 30% of the channels were in state  $C^*$ , and 70% in  $C'^*$ . It was presumed that transitions from  $C^*$  to  $O^*$  and from

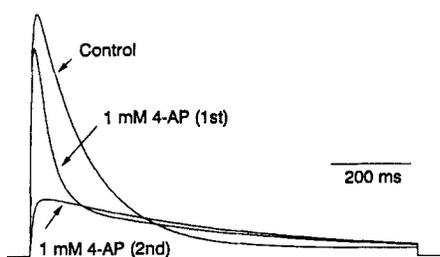


FIGURE 11. Computer simulation of the action of 4-AP. Family of computer simulated currents in control conditions and during the first and the second pulses after addition of 1 mM 4-AP. It was postulated that both open and inactivated channels can be blocked with a forward rate constant of  $15 \text{ s}^{-1}$ , and unblocked at  $7 \text{ s}^{-1}$ . At the end of the first pulse in the pres-

ence of the drug, 64% of the channels are blocked, thus producing the marked reduction in the amplitude of the second pulse. The rate of channel inactivation was made equal to  $7.5 \text{ s}^{-1}$ .

$C'^*$  to  $O'^*$  were fast ( $200\text{ s}^{-1}$ ), while those from  $O^*$  to  $O$  and from  $O'^*$  to  $O$  were, respectively, 30 and  $2\text{ s}^{-1}$ . 98% of the channels were unblocked at the end of this theoretical pulse, in agreement with the experimental data.

### Conclusion

A number of studies have investigated the mechanism of the blocking effect of 4-AP on  $K^+$  channels present on nerve cells. This block is different for different channel types (Dubois, 1981), it is potential dependent, and it is removed at positive potentials (Ulbricht and Wagner, 1976; Yeh et al., 1976; Meves and Pichon, 1977). These results were interpreted as indicating that 4-AP binds to closed channels and is released from open channels in a voltage-dependent manner (Yeh et al., 1976). In a study on the action of 4-AP on the transient outward current of a molluscan central neuron, Thompson (1982) proposed a model according to which 4-AP binds to closed and open channels, while no transitions occur between the inactivated and blocked states. A similar scheme has been proposed for the transient outward current of the heart (Simurda et al., 1989). Both studies suggested that the relief of block upon depolarization is due to the lack of binding of 4-AP to inactivated channels. Our results show that 4-AP does not block  $K^+$  channels of lymphocytes in exactly the same way.

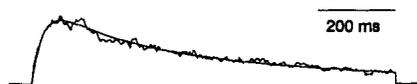


FIGURE 12. Computer simulation of drug washout. Superposition of a computer-simulated curve obtained

using the model presented in the text, with an experimental recording of the current elicited by the first depolarizing pulse after wash-out of 4-AP. Settings: at rest the population of channels was distributed between two closed blocked states  $C'^*$  and  $C^*$ . Upon cell depolarization they evolve toward two activated blocked states,  $O'^*$  and  $O^*$ , respectively, with equal rate constants of  $200\text{ s}^{-1}$ , and then proceed to the open state,  $O$ , with rate constants of 30 and  $2\text{ s}^{-1}$ , respectively. The rate of channel inactivation was made equal to  $7.5\text{ s}^{-1}$ .

A major difference in our results is the absence of relief of the block upon cell depolarization. Rather, when rapidly applying and removing drugs, we found that lymphocytes need to be depolarized in order for the block and its relief to occur. These results were interpreted in terms of a model in which 4-AP binds to open channels, and cannot bind to, or unbind from, closed channels. Furthermore, the observation that the steady-state block is established after application of a single depolarizing pulse is consistent with the notion that 4-AP binds to inactivated channels. This property would be sufficient by itself to account for the different voltage dependence of the block exhibited by  $K^+$  channels in neurones and lymphocytes.

Thus, a common feature of the action of 4-AP in all cells is that it depends upon the kinetic state of the channel; however, marked differences are found in the particular state of the channel that is sensitive to 4-AP. It only blocks the closed state of transient  $K^+$  channels of rat melanotrophs (Kehl, 1990), only acts after channel opening in lymphocytes (this study) or GH3 pituitary cells (Wagoner and Oxford, 1990), and affects both open and closed channels in invertebrate neurones (Thomp-

son, 1982). Despite this heterogeneity, some of our results, such as the reduction by 4-AP of the burst open time, can probably be extended to  $K^+$  channels of other systems.

A number of studies indicated that 4-AP blocks the channels when applied inside or outside the cell (Yeh et al., 1976; Thompson, 1982), while others (Herman and Gorman, 1981) observed that 4-AP is more effective when applied inside the cell. These data are consistent with our hypothesis that 4-AP can cross the membrane and then block the channels from the internal side. This property, revealed by the pH dependence of 4-AP action, is of general importance in investigating the nature (ionized versus nonionized) of the compound that acts on the channel, and thus its likely site of action.

Our results show that the discrepancy between the  $K_d$  for channel blockade and cell function in lymphocytes cannot be accounted for by the voltage dependence of 4-AP binding. This discrepancy might result from the fact that the cell incubation medium often becomes acidic after a few days in culture, and that 4-AP is less potent at acidic extracellular pH. However, against this hypothesis, both verapamil and quinine block cell function and  $K^+$  channels with the same potency (Amigorena et al., 1990), although the effects of these blockers also depend upon pH (Choquet, D., personal observations).

The absence of binding of 4-AP to closed channels is also unlikely to account for the discrepancy between blockade of channels and cell function. Tetraethylammonium only blocks open channels (Grissmer and Cahalan, 1989b) and is equally potent on channel and cell function (Amigorena et al., 1990). Alternatively, lymphocytes might express a class of voltage-independent  $K^+$  channels that are insensitive to 4-AP.

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