

The Calcium-independent Transient Outward Potassium Current in Isolated Ferret Right Ventricular Myocytes

II. Closed State Reverse Use-dependent Block by 4-Aminopyridine

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ABSTRACT Block of the calcium-independent transient outward K^+ current, I_{to} , by 4-aminopyridine (4-AP) was studied in ferret right ventricular myocytes using the whole cell patch clamp technique. 4-AP reduces I_{to} through a closed state blocking mechanism displaying "reverse use-dependent" behavior that was inferred from: (a) development of tonic block at hyperpolarized potentials; (b) inhibition of development of tonic block at depolarized potentials; (c) appearance of "crossover phenomena" in which the peak current is delayed in the presence of 4-AP at depolarized potentials; (d) relief of block at depolarized potentials which is concentration dependent and parallels steady-state inactivation for low 4-AP concentrations ($V_{1/2} \approx -10$ mV in 0.1 mM 4-AP) and steady-state activation at higher concentrations ($V_{1/2} = +7$ mV in 1 mM 4-AP, $+15$ mV in 10 mM 4-AP); and (e) reassociation of 4-AP at hyperpolarized potentials. No evidence for interaction of 4-AP with either the open or inactivated state of the I_{to} channel was obtained from measurements of kinetics of recovery and deactivation in the presence of 0.5–1.0 mM 4-AP. At hyperpolarized potentials (–30 to –90 mV) 10 mM 4-AP associates slowly (time constants ranging from ~800 to 1,300 ms) with the closed states of the channel (apparent $K_d \approx 0.2$ mM). From –90 to –20 mV the affinity of the I_{to} channel for 4-AP appears to be voltage insensitive; however, at depolarized potentials (+20 to +100 mV) 4-AP dissociates with time constants ranging from ~350 to 150 ms. Consequently, the properties of 4-AP binding to the I_{to} channel undergo a transition in the range of potentials over which channel activation and inactivation occurs (–30 to +20 mV). We propose a closed state model of I_{to} channel gating and 4-AP binding kinetics, in which 4-AP binds to three closed states. In this model 4-AP has a progressively lower affinity as the channel approaches the open state, but has no intrinsic voltage dependence of binding.

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INTRODUCTION

Since the initial demonstration by Pelhate and Pichon (1974) that 4-aminopyridine (4-AP) blocked the delayed rectifier K^+ current of cockroach axons, aminopyridines have served as pharmacological tools for the study and classification of K^+ channels in various tissues (e.g., Glover, 1982; Lechat, Thesleff, and Bowman, 1982; Rudy, 1988; Pelhate and Malecot, 1989). In particular, block by 4-AP is often used as an indicator for identifying voltage-dependent inactivating " I_A " or " I_{to} " K^+ currents. Furthermore, in the absence of more specific compounds 4-AP has been widely used in studies on mammalian cardiac myocytes as a pharmacological agent for separating I_{to} into two separate current components, a larger, voltage-activated, 4-AP-sensitive " I_{to1} " and a smaller, calcium-activated " I_{to2} " (e.g., Binah, 1990; Gintant, Cohen, Datyner, and Kline, 1991). However, preliminary accounts have suggested that block of cardiac I_{to1} by 4-AP displays use-dependent characteristics (e.g., dog ventricle: Simurda, Simurdova, and Christe, 1989). Despite its widespread use in studies of K^+ currents in various cardiac myocytes, the mechanism of block of cardiac I_{to1} by 4-AP has not been quantitatively characterized to date.

In this article we describe the quantitative characteristics of blockade by 4-AP of the calcium-insensitive I_{to} ($= I_{to1}$) in ferret enzymatically isolated right ventricular myocytes (Campbell, Rasmusson, Qu, and Strauss, 1993). We present data indicating that block by 4-AP displays strong reverse use-dependent characteristics (cf. Hondeghem and Snyders, 1990); i.e., block is relieved at depolarized potentials or by rapid rates of stimulation. Our measurements suggest that 4-AP does not interact strongly with either the open or inactivated state of the I_{to} channel, but rather produces block through a closed state binding mechanism. These interactions lead to an alteration of the kinetics of I_{to} in the presence of 4-AP. These measurements are combined with the characterization of I_{to} gating kinetics presented in the preceding paper (Campbell et al., 1993) to demonstrate one possible multiple closed state binding model that is able to reproduce quantitatively the experimentally observed characteristics of 4-AP block. The simulations presented suggest that such a multiple closed state binding mechanism could serve as a useful general model for various compounds that appear to display reverse use-dependent blocking characteristics of cardiac K^+ channels (cf. Hondeghem and Snyders, 1990).

Preliminary accounts of this work have appeared in abstract form (Campbell, Qu, Rasmusson, and Strauss, 1991*a, b*, 1992; Strauss, Campbell, Rasmusson, and Qu, 1992).

METHODS

All experiments were conducted on myocytes enzymatically isolated from the right ventricles of 10–16-wk-old male ferrets using the whole cell configuration of the gigaseal patch clamp technique. Myocyte isolation, electrophysiological recording techniques, experimental solutions, and statistical, analytical, and numerical methods were as described in the preceding paper (Campbell et al., 1993). All experiments were conducted in Na^+ -free, *N*-methyl-D-glucamine (NMDG) solution (12–20 μ M tetrodotoxin, 500 μ M Cd^{2+} ; pH 7.40) and at room temperature (21–23°C). 4-AP (Aldrich Chemical Co., Milwaukee, WI) was added directly (0.0625–10 mM) to NMDG saline. pH was then readjusted (with HCl) back to pH 7.40. Since 4-AP is an organic base (pK_a 9.2; Albert, Goldacre, and Philips, 1948), >98% of it existed in

the charged cationic form (4-AP⁺) in the NMDG recording solutions used. To allow adequate reequilibration of 4-AP with its binding sites (see Results), all voltage clamp protocols (both single and double pulse protocols) were applied (depending on the concentration of 4-AP used) at an appropriately low frequency ranging from one pulse protocol per 10 s to one pulse protocol per 2–4 min.

For ease of discussion, the calcium-insensitive transient K⁺ current will be referred to simply as I_{to} (see Campbell et al., 1993). Unless otherwise indicated, all data are given as mean values \pm SD.

RESULTS

Basic Observation: 4-AP Both Reduces Peak I_{to} and Alters Its Apparent Kinetics

In the preceding paper (Campbell et al., 1993) it was noted that application of 4-AP both reduced the rapid, early, "phase 1" repolarization and prolonged the duration of the action potential plateau (Fig. 1 *A* of Campbell et al., 1993). The effects of 4-AP on I_{to} were investigated further under voltage clamp conditions. Fig. 1 shows the effects of 5 mM 4-AP on I_{to} in a single ferret right ventricular myocyte elicited by an 800-ms voltage clamp pulse to +50 mV. 5 mM 4-AP reduced I_{to} appreciably,

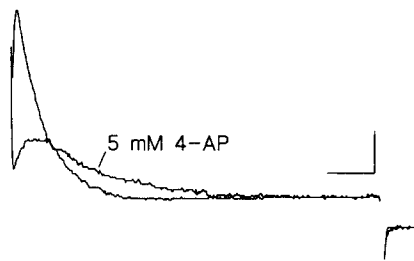


FIGURE 1. Effect of 4-AP on I_{to} . 800-ms voltage clamp pulses were applied to +50 mV (HP = -70 mV) in control NMDG saline and 5 mM 4-AP. 4-AP not only reduced peak I_{to} but also slowed the activation and time course of current decay, resulting in a crossover of the current waveforms. Calibration: 100 ms, 100 pA.

consistent with its observed effects on the action potential. However, 4-AP not only reduced peak I_{to} but also slowed the apparent rates of both activation and inactivation. As a result, there is a crossover of the current waveforms recorded before and after application of 4-AP.

4-AP Dose-Response Relationship

The dose-response relationship for 4-AP was measured over the range of depolarized potentials at which I_{to} is activated. For each myocyte studied the control peak I_{to} current-voltage (I - V) relationship was first determined from +20 to +100 mV (500-ms clamp pulses; 10-mV increments; holding potential [HP] = -70 mV). This protocol was repeated with progressively increasing concentrations of 4-AP (range: 0.0625–10 mM). The dose-response relationship at each test potential was then expressed as the percent reduction in control peak I_{to} as a function of 4-AP concentration (peak currents were measured relative to the holding current and were not leakage corrected; see Campbell et al., 1993). To allow reequilibration of 4-AP between depolarizing pulses (see below), at low concentrations of 4-AP (0.0625–1

mM) a frequency of one pulse per 1–2 min was used; at higher concentrations (> 1 mM) pulse frequency was increased (one pulse per 10–30 s).

At all depolarized potentials reduction in peak I_{to} as a function of 4-AP concentration could be described by a single binding site equation that provided an apparent K_d (i.e., the concentration of 4-AP required to produce 50% maximal block of peak I_{to}). A representative result of this analysis is illustrated in Fig. 2 *A*, which shows the dose–response relationship obtained at +50 mV (based on $n = 2$ –8 myocytes in

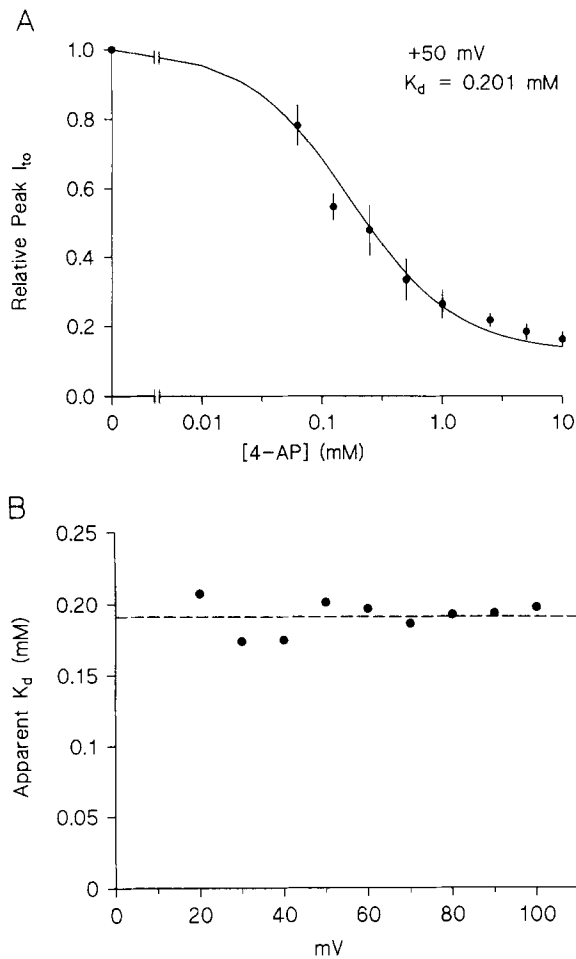


FIGURE 2. Dose–response relationship for block of peak I_{to} by 4-AP. (*A*) Representative dose–response relationship obtained at +50 mV from 500-ms I - V curves obtained in control and varying concentrations of 4-AP (0.0625 to 10 mM; see text). The curve was constructed as the mean reduction in control peak I_{to} at +50 mV as a function of applied [4-AP]. The data points (mean values from $n = 5$ –6 myocytes at each [4-AP]) were fit (smooth curve) to a single binding site equation $(I_{to,4-AP}/I_{to,control}) = 1/(1 + [4-AP]/K_d)$, which incorporated a correction for the background leak current with a derived best-fit apparent K_d of 0.201 mM. (*B*) Potential dependence (+20 to +100 mV) of the apparent K_d for block of peak I_{to} . The apparent K_d was independent of membrane potential, giving an overall mean apparent K_d of 0.191 ± 0.011 mM (dashed line).

different concentrations of 4-AP). Analysis of the data points obtained at +50 mV gave a best-fit apparent K_d value of 0.201 mM (smooth curve fit). The K_d potential relationship derived over the potential range +20 to +100 mV was independent of applied pulse potential. As a result, the K_d values extracted at each potential were averaged, giving an overall mean apparent K_d of 0.191 ± 0.011 mM (dashed line in Fig. 2 *B*).

Can 4-AP Produce Tonic Block of I_{to} ?

A recent study on the effects of 4-AP on transient K^+ channels in mouse lymphocytes indicated that 4-AP could not bind to channels maintained in their resting state; i.e., tonic block could not develop at hyperpolarized potentials (Choquet and Korn, 1992; see Discussion). To determine if 4-AP could produce tonic block of I_{to} in ferret ventricular myocytes, cells were initially clamped to either $HP = 0$ mV or $HP = -70$ to -90 mV. After 2 min at either initial HP, a 150-ms pulse to -70 mV was applied, followed by a 500-ms pulse to $+50$ mV (see schematic inset in Fig. 3 B; the 150-ms gap to -70 mV was applied before the pulse to $+50$ mV to allow recovery from inactivation produced at $HP = 0$ mV). After returning to either 0 or -70 to -90 mV, 0.1 mM 4-AP was perfused for 2–4 min without any pulsing. At the end of this 2–4-min tonic perfusion period the 500-ms pulse to $+50$ mV was again applied to assess the degree of block that developed at the initial HP.

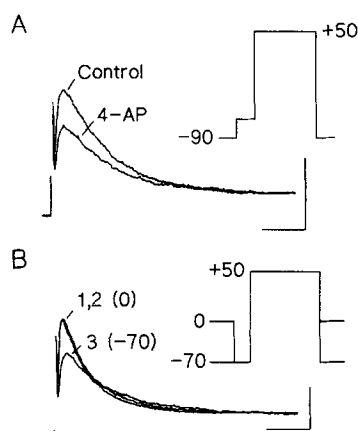


FIGURE 3. Potential dependence of tonic block of I_{to} by 4-AP. Voltage clamp protocols are shown schematically in inset; see text for further details. (A) 4-AP produced tonic block at $HP = -90$ mV. I_{to} was elicited at $+50$ mV in control and after 4 min of tonic perfusion of 0.1 mM 4-AP. Calibration: 50 ms, 500 pA. (B) 4-AP did not produce tonic block at $HP = 0$ mV. I_{to} recordings 1 and 2 were elicited at $+50$ mV from $HP = 0$ mV first in control (1) and then after 4 min of tonic perfusion of 0.1 mM 4-AP (2) at $HP = 0$ mV. The current traces superimposed, indicating that 4-AP did not

produce tonic block. The myocyte was then clamped to $HP = -70$ mV for an additional 4 min of 0.1 mM 4-AP tonic perfusion, after which 4-AP blocked I_{to} elicited at $+50$ mV (3). Calibration: 50 ms, 400 pA.

Fig. 3 shows results obtained from two different myocytes where this protocol was applied. In Fig. 3 A the myocyte was initially held at $HP = -90$ mV. At the end of a 2-min tonic perfusion period 0.1 mM 4-AP produced measurable block. In contrast, Fig. 3 B shows results obtained from another myocyte that was initially held at $HP = 0$ mV, a potential that produces $<3\%$ activation but 92% inactivation (Campbell et al., 1993). In this case, at the end of 4 min of tonic perfusion 0.1 mM 4-AP did not block I_{to} . However, when the same myocyte was subsequently stepped to -70 mV for an additional 4 min of tonic perfusion, significant block was produced. These results indicate that I_{to} channels do not have to be previously activated in the presence of 4-AP for block to develop. Such tonic block develops only at hyperpolarized potentials, with little or no block developing at 0 mV (cf. Choquet and Korn, 1992).

We conclude from these observations that 4-AP binds to the closed (resting) state(s), but does not bind significantly to the inactivated state(s) of the I_{to} channel.

Potential Dependence of Relative Block by 4-AP

The dose-response analysis summarized in Fig. 2 indicates that the degree of block of peak I_{to} produced by a given concentration of 4-AP is independent of the pulse potential applied to activate I_{to} (+20 to +100 mV). However, the tonic block experiments illustrated in Fig. 3 indicate that development of block depends on holding potential. To determine further the effect of varying membrane potential on the degree of block produced by 4-AP at a fixed depolarized potential, a double pulse P1-P2 protocol was applied (see schematic inset in Fig. 4 B). From a fixed holding potential of -70 mV, a 500-ms depolarizing P1 pulse was applied in 10-mV increments. Membrane potential was returned to -70 mV for 100 ms, and then a fixed P2 pulse of 500 ms was applied to +50 mV. This protocol was first conducted in control NMDG saline and then repeated in 1 and/or 10 mM 4-AP.

Fig. 4 A shows a typical result obtained using this protocol before and after perfusion of 10 mM 4-AP. In control, as P1 was made more depolarized, the I_{to} during P2 progressively decreased to a final steady-state value (as would be predicted for inactivation produced by P1 and subsequent recovery during the 100-ms gap back to -70 mV; see Campbell et al., 1993). However, when the protocol was repeated in 10 mM 4-AP, the behavior of the P2 I_{to} waveforms was radically altered. For small to moderate P1 depolarizations the I_{to} current during P2 was small, as would be predicted for block in 10 mM 4-AP at HP = -70 mV. However, as P1 became progressively more depolarized (> 0 mV) the P2 I_{to} began to both increase in size and display crossover (cf. Fig. 1).

The results obtained using this double pulse P1-P2 protocol in 1 and 10 mM 4-AP are summarized in Fig. 4 B. In this figure the data have been normalized as relative percent maximum block of I_{to} during P2 as a function of P1 potential (i.e., relative maximum block for P1 = -70 mV [control] was defined as 100% block; relative minimum block for P1 = +70 mV was defined as 0% block). The degree of block produced by 4-AP is markedly potential dependent, with maximum block occurring at hyperpolarized potentials and minimum block at depolarized potentials. The relative block-membrane potential relationship also depends on 4-AP concentration: increasing 4-AP from 1 to 10 mM shifts the relationship in the depolarized direction. The potential at which half-maximal relative block occurred was approximately +7 mV in 1 mM and +15 mV in 10 mM 4-AP.

One hypothesis for interpretation of the data illustrated in Fig. 4 would be that 4-AP dissociates from its binding site(s) on or in the I_{to} channel protein at depolarized potentials, and associates with its binding site(s) at hyperpolarized potentials. Since the channel would be in one of its closed states at hyperpolarized potentials, the data in Fig. 4 suggest that 4-AP binds to the closed state(s).

Kinetic Analysis of Association of 4-AP

The kinetics of 4-AP associating at hyperpolarized potentials were examined by a conventional double pulse P1-P2 recovery protocol applied in the presence of 4-AP (see schematic inset in Fig. 5 B). From a fixed holding potential, identical 500 ms P1

and P2 pulses, separated by a variable interpulse interval Δt , were applied to +50 mV; Δt was then progressively increased and the resultant I_{to} during P2 was analyzed.

Fig. 5 A shows an example of an I_{to} recovery waveform during P2 obtained in the presence of 10 mM 4-AP (HP = -60 mV). For clarity, the figure only shows selected recordings for Δt of 200 ms and greater. The behavior of the I_{to} recovery waveform in 4-AP was highly unconventional. At $\Delta t = 200$ ms, I_{to} during P2 was larger than I_{to} during P1; however, as P1 and P2 were separated by progressively increasing Δt , I_{to} during P2 slowly declined back to the control P1 level. Fig. 5 B summarizes the time

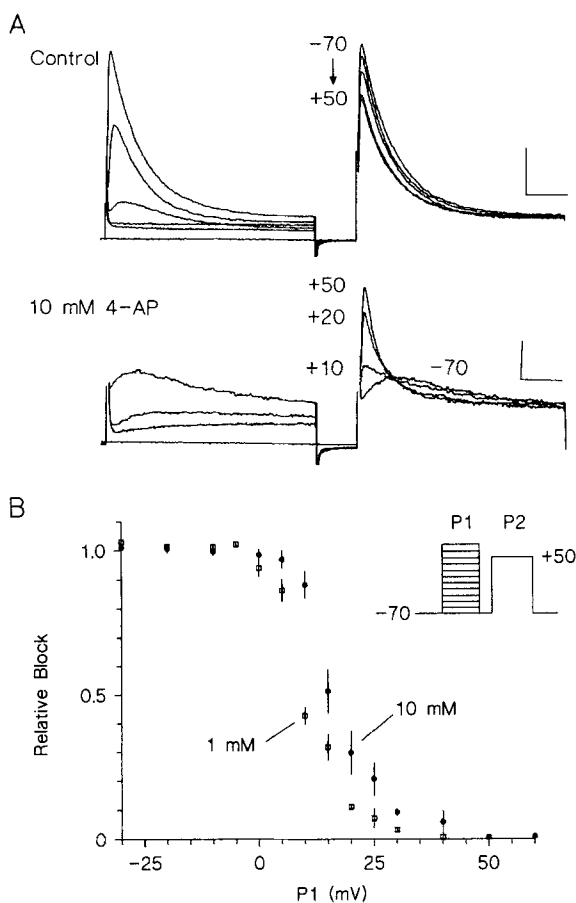


FIGURE 4. Voltage dependence of 4-AP block of I_{to} . Voltage clamp protocol is shown schematically in inset. (A) Representative I_{to} currents obtained before (control: P1 = -70, -50, -20, +10, +30, +50 mV) and after application of 10 mM 4-AP (P1 = -70, +10, +20, +50 mV). Note that in 10 mM 4-AP the I_{to} during the fixed P2 to +50 mV increased at depolarized P1 potentials, indicating that block of 4-AP was relieved at depolarized potentials. The kinetics of the P2 I_{to} waveforms were also altered, producing crossover behavior. Calibration: control, 100 ms, 400 pA; 4-AP, 100 ms, 200 pA. (B) Potential dependence of relative block (see text) of I_{to} by 1 and 10 mM 4-AP (data points are mean values from $n = 6$ myocytes at each concentration).

course of the relative P2 I_{to} data obtained from the experiment shown in Fig. 5 A for the same recovery pulse protocol applied from two different holding potentials (-60 and -90 mV). The decline of P2 I_{to} could be reasonably described as a single exponential process.

The data presented in Fig. 5, A and B are consistent with the hypothesis that 4-AP dissociates from the I_{to} channel during P1 to +50 mV, and slowly reassociates with the closed channel during the interpulse interval Δt with a time constant that depends on holding potential. Using the double pulse recovery protocol described in

Fig. 5 *B*, the kinetics of association of 10 mM 4-AP with the closed states of the I_{to} channel were determined over the holding potential range of -90 to -30 mV. The summarized time constants of association, τ_{assoc} , are given in Fig. 5 *C*. The mean value of τ_{assoc} increases with increasing hyperpolarization (from 808 ± 84 ms at -30 mV to $1,338 \pm 84$ ms at -90 mV).

Two mechanisms could account for the increase of τ_{assoc} with increasing hyperpolarization. First, the affinity for 4-AP of the closed I_{to} channel could progressively

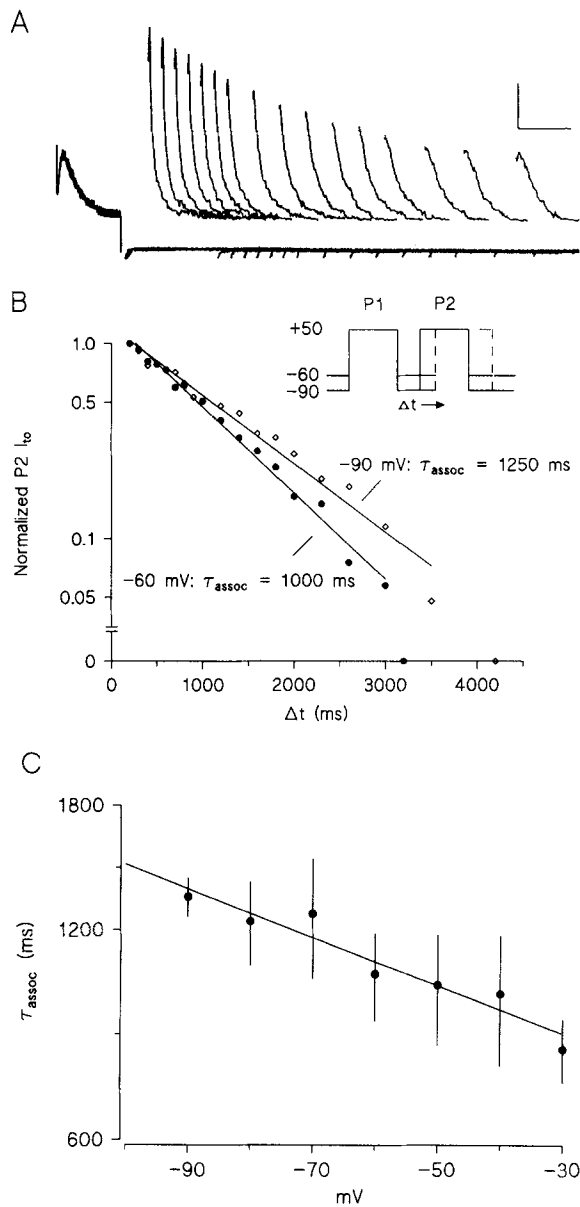


FIGURE 5. Association of 10 mM 4-AP at hyperpolarized potentials. (*A*) Representative bi-phasic recovery behavior in 10 mM 4-AP obtained at HP = -60 mV using a 500-ms double pulse recovery protocol (see schematic inset in *B*). Current traces obtained with an inter-pulse interval of $\Delta t = 200$ ms and greater are illustrated. Calibration: 400 ms, 100 pA. (*B*) Kinetics of 4-AP association at HP = -60 and -90 mV for the myocyte illustrated in *A*. At both potentials 4-AP association could be reasonably described by an exponential process (τ_{assoc} 's indicated). (*C*) Potential dependence (HP = -30 to -90 mV) of the time constants of 4-AP association, τ_{assoc} . Data obtained in 10 mM 4-AP using the 500-ms double pulse recovery protocol illustrated in *B*. Mean values from four different myocytes at each HP. Mean data points were best fit (straight line) with the following equation: $\tau_{\text{assoc}} = 671 \exp(-0.008 \cdot V)$ (V in millivolts).

decrease with increasing hyperpolarization. This could occur if the different closed states of the channel (Campbell et al., 1993) displayed different K_d values for 4-AP binding, with the closed state immediately adjacent to the open state C_1 having the highest affinity, the intermediate closed state C_2 an intermediate affinity, and the first closed state C_3 the lowest affinity. Hyperpolarization would favor the lower affinity closed states, thereby causing τ_{assoc} to increase. If such a decreasing affinity mechanism is applicable, then at a low concentration of 4-AP ($\leq K_d$) the degree of block produced at a fixed depolarized potential should systematically decrease as holding potential is made more hyperpolarized. Alternatively, the affinities of all the closed states for 4-AP could be identical or approximately equal, but the effective turnover rates ($K_d = k_{off}/k_{on}$) could differ. This could arise, for example, from a voltage-

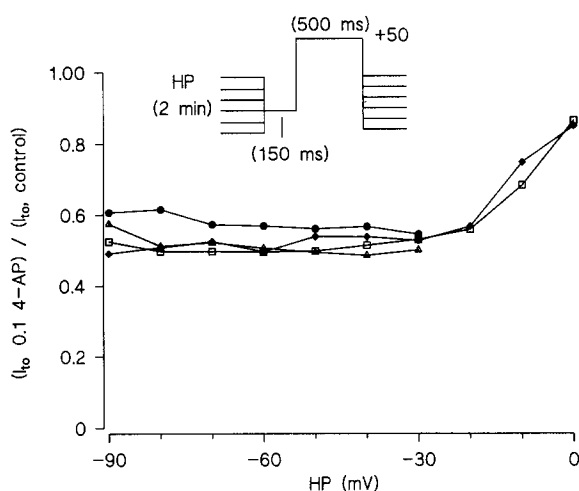


FIGURE 6. Block of I_{to} by 0.1 mM 4-AP as a function of holding potential (-90 to 0 mV). Voltage clamp protocol is shown in the schematic inset; see text for details. Data points (from four different myocytes) show the relative block of peak I_{to} at $+50$ mV produced by 0.1 mM 4-AP from HP = -90 to 0 mV. The pulse protocol was applied once every 2 min from each HP first in control and then in 0.1 mM 4-AP. From -90 to -30 mV the degree of block was potential independent, but block was progressively relieved by depolarization positive to approximately -20 mV, where activation and inactivation are overlapping processes.

dependent conformational change, either in the channel protein or its immediate environment, that somehow restricts the access and exit of 4-AP to its binding sites without altering the affinity of the binding sites themselves. In this restricted access case, the degree of block produced by a low concentration of 4-AP could remain relatively constant as holding potential is varied.

To test between these two possibilities the following voltage clamp protocol was applied (see schematic inset in Fig. 6). In control NMDG saline, myocytes were first held at a variable holding potential (range 0 to -90 mV), pulsed to -70 mV for 150 ms, and then pulsed to $+50$ mV for 500 ms to elicit a control I_{to} . This protocol was then repeated in 0.1 mM 4-AP at a frequency of one pulse protocol per 2 min. As shown in Fig. 6 (which illustrates pooled results obtained from four myocytes) the degree of block of peak control I_{to} by 0.1 mM 4-AP remained constant as HP was

varied from -90 to -20 mV. Only when holding potential was depolarized beyond -20 mV was block reduced. These results indicate that the closed states C_3 and C_2 (existing from -90 to -20 mV) exhibit affinities for 4-AP that are not substantially different. However, the relief of block produced by depolarization above -20 mV results from an aggregate of activation and inactivation, as well as drug affinity to the C_1 state. The present data therefore do not quantitatively define the affinity of 4-AP for the C_1 state.

Kinetic Analysis of Dissociation of 4-AP

The rate at which 4-AP dissociates from the I_{to} channel at depolarized potentials was quantified using the protocol schematically illustrated in the inset in Fig. 7 B. From a fixed holding potential (HP = -70 mV) and in the presence of 10 mM 4-AP, a variable duration P1 was applied to a given depolarized potential ($+20$ to $+100$ mV). After a return gap of 100 ms back to the holding potential, a 500-ms P2 was applied to $+50$ mV. The 100-ms gap was required to allow for recovery from inactivation produced during P1, while allowing only minimal reassociation of 4-AP (refer to Fig. 5). The duration of P1 at a given fixed potential was then progressively increased. If the hypothesis that 4-AP dissociates during P1 is correct, then block of I_{to} during P2 should be lessened with increasing P1 duration: the amplitude of the P2 I_{to} waveform during P2 should progressively increase with P1 duration, displaying a time course that reflects the kinetics of 4-AP dissociation during P1.

Fig. 7 A shows a representative example of the P2 I_{to} waveform generated by applying this protocol to a myocyte in 10 mM 4-AP. For this particular experiment P1 was stepped to $+50$ mV and its duration, Δt , was incremented by 50–100-ms steps. As Δt increased (and I_{to} during P1 progressively inactivated), the I_{to} during P2 progressively increased until a final saturating value was reached after several hundreds of milliseconds. These results demonstrate that 4-AP is progressively dissociating during the P1 pulse to $+50$ mV. Fig. 7 B shows the time course of the normalized I_{to} during P2 for this same myocyte at three different P1 potentials ($+30$, $+50$, and $+70$ mV). The time course of dissociation at all three P1 potentials could be described as a single exponential process with voltage-dependent time constants, τ_{dissoc} , that decreased with depolarization. For the myocyte illustrated, the values of τ_{dissoc} were 280 ms at $+30$ mV, 230 ms at $+50$ mV, and 167 ms at $+70$ mV.

The dependence of τ_{dissoc} on membrane potential over the range $+20$ to $+100$ mV in 10 mM 4-AP is given in Fig. 7 C (based on $n = 4$ –5 myocytes at each potential). 4-AP dissociates faster with increasing depolarization (range: $\tau_{dissoc} = 349 \pm 70$ ms at $+20$ mV to 133 ± 14 ms at $+100$ mV).

In summary, the kinetic measurements indicate that in the hyperpolarized range of potentials, 4-AP (10 mM) associates with the closed state(s) of the I_{to} channel with time constants on the order of 1 s, with association becoming slower with increasing hyperpolarization. Over the depolarized range of potentials, 4-AP dissociates with time constants on the order of hundreds of milliseconds, with dissociation becoming faster with increasing depolarization.

Does 4-AP Bind to Channel States Other Than Closed Ones?

The experimental results presented indicate that 4-AP binds to the closed states of the I_{to} channel. Two additional series of experiments were conducted to explore the possibility that 4-AP also interacts with either (a) the inactivated state and/or (b) the open state of I_{to} channels.

Inactivated state binding? Two sets of experimental results presented in the preceding sections suggest that 4-AP does not bind to the final inactivated state of I_{to}

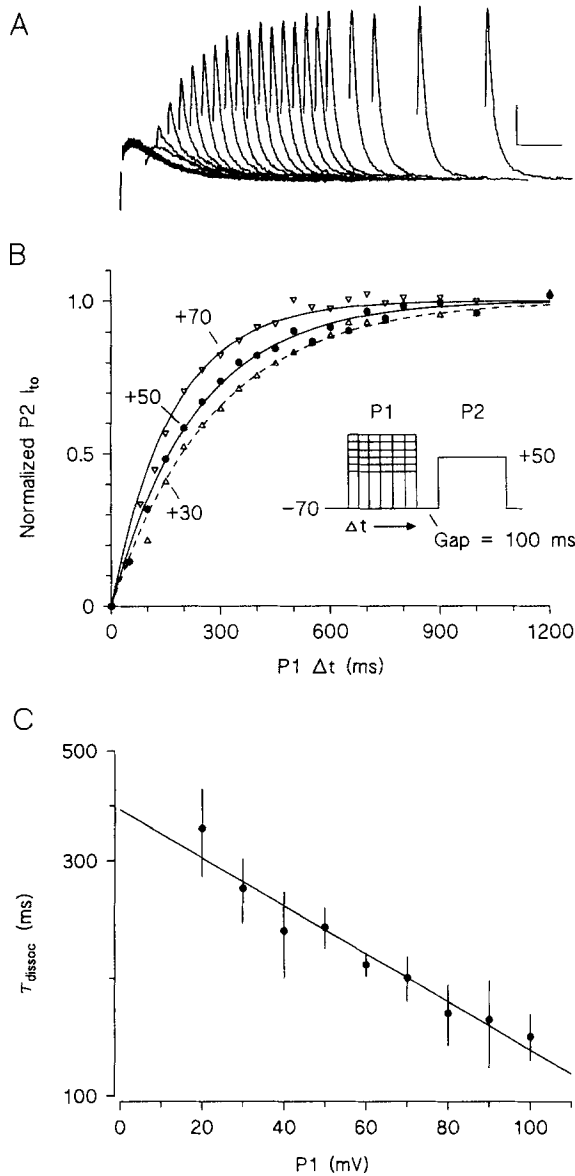


FIGURE 7. Kinetics of dissociation of 4-AP at depolarized potentials. Voltage clamp protocol is illustrated in the schematic inset in *B*. The gap of 100 ms allowed sufficient recovery of I_{to} to occur with only a minimal amount of 4-AP reassociation (see Fig. 5). (*A*) Representative demonstration of 4-AP dissociation at P1 = +50 mV (increasing P1 duration in initial increments of 50–100 ms). Note that with increasing P1 duration, I_{to} during P2 progressively increased, indicating that 4-AP was dissociating during P1 with kinetics reflected by the envelope of the currents during the P2 waveform. Calibration: 200 ms, 200 pA. (*B*) Kinetics of 4-AP dissociation for the myocyte illustrated in *A* obtained at P1 potentials of +30, +50, and +70 mV. For all P1 potentials dissociation could be reasonably described as an exponential process. (*C*) Potential dependence of time constants of 4-AP dissociation, τ_{dissoc} , obtained in 10 mM 4-AP using the voltage clamp protocol illustrated in *B*. Mean data points for $n = 4-5$ myocytes at each P1 potential. Mean data points were best fit (solid line) with the following equation: $\tau_{dissoc} = 381 \exp(-0.00112 \cdot V)$ (V in millivolts).

channels. First, tonic block did not develop at 0 mV (Fig. 3B), where 92% of I_{to} channels are inactivated (Campbell et al., 1993). Second, in our dissociation measurements in 10 mM 4-AP (e.g., Fig. 7A), the amplitude of P2 I_{to} increased with increasing P1 duration, even though the percentage of channels entering the final inactivated state progressively increased as P1 duration increased. However, we performed additional experiments to search for behavior that was inconsistent with a pure closed state blocking mechanism.

If observed, a slowing of the kinetics of recovery from inactivation by 4-AP would be evidence for binding to the inactivated state and would be inconsistent with a pure closed state blocking mechanism. To determine the possible effects of 4-AP on the kinetics of recovery from inactivation, double pulse recovery protocols were conducted in control NMDG and 1 mM 4-AP using the same protocol previously described in Fig. 5. These experiments were identical to those used to measure the kinetics of association, except that the initial time course of recovery from inactivation

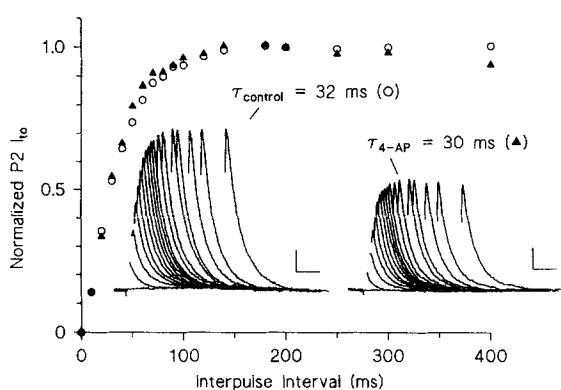


FIGURE 8. Effect of 4-AP on the kinetics of I_{to} recovery from inactivation. The inset shows representative I_{to} recovery waveforms during P2 to +50 mV obtained in control and 1 mM 4-AP from HP = -70 mV (see Fig. 5 for recovery voltage clamp protocol). The main figure illustrates the time course of recovery of the normalized P2 currents illustrated in the inset. Due to the association of 4-AP during the interpulse interval

Δt , the P2 currents have been normalized to their peak values at 200 ms. Using this protocol, the kinetics of recovery from inactivation were not altered by 4-AP (recovery time constants: control, 32 ms; 1.0 mM 4-AP, 30 ms). Calibration: 90 ms, 200 pA.

tion of the I_{to} current during P2 was analyzed (interpulse interval $\Delta t = 10$ –200 ms, i.e., before significant reassociation of 4-AP had developed).

Fig. 8 shows results of such a recovery protocol (HP = -70 mV) applied to a myocyte bathed first in control NMDG saline and then in 1 mM 4-AP. The time constants of recovery from inactivation, as determined during the first 200 ms, were the same in control (32 ms) and 4-AP (30 ms) solutions. Similar results were obtained from a total of three myocytes in 1 mM 4-AP at two different holding potentials (HP = -70 mV: $\tau_{\text{control}} = 35 \pm 4$ ms, $\tau_{4\text{-AP}} = 34 \pm 5$ ms; HP = -90 mV: $\tau_{\text{control}} = 22 \pm 6$ ms, $\tau_{4\text{-AP}} = 24 \pm 5$ ms). These results are consistent with the hypothesis that 4-AP does not bind to the final inactivated state of unblocked channels.

Open state binding? Two sets of experimental observations have been used previously as evidence that 4-AP can produce rapid open channel block of I_{to} -like currents in certain tissues: (a) a decrease in the time to peak current and the appearance of an early rapid phase of inactivation (e.g., molluscan neurons: Thomp-

son, 1982); and (b) an acceleration of the overall apparent rate of inactivation (e.g., cloned *Drosophila* H4 channels expressed in *Xenopus* oocytes: Hice, Swanson, Folander, and Nelson, 1992). In contrast, we consistently observed a concentration-dependent (0.0625–10 mM) increase in the time to peak current and a slowing of apparent inactivation.

The open channel blocking characteristics described above are essentially due to rapid binding and very slow unbinding of the blocking compound to the channel pore binding site. Other K^+ channel blockers display an open channel block/unblock that is rapid compared with the mean channel open time, resulting in a rapid "flickering block" (e.g., Moczydlowski, 1986; Hille, 1992). When the binding of such a rapid flickering blocking compound prevents channel closing or inactivation from developing, the result is a "burst" of longer duration than the normal channel open time. If such a mechanism of block occurs in ferret ventricular I_{to} channels, then it would be expected that the time constants of deactivating tail currents will be prolonged in the presence of 4-AP (e.g., similar to that observed for open channel block of the cardiac delayed rectifier current by quinidine: Colatsky, Follmer, and Starmer, 1990; Snyders, Knoth, Roberds, and Tamkun, 1992).

To examine possible changes in deactivation kinetics for evidence of open channel interaction, a modified tail current protocol was applied in control NMDG and 0.5 mM 4-AP. From a constant HP = -70 mV a brief (10–25 ms) P1 pulse to a depolarized potential (+50 to +70 mV) was immediately followed by a P2 pulse to a hyperpolarized potential (0 to -20 mV) so as to generate a control P2 I_{to} tail current. After perfusing 0.5 mM 4-AP at -70 mV for 1–2 min (to allow equilibration), the pulse protocol was repeated 20–25 times at a rapid frequency (1–1.4 Hz). Due to progressive dissociation of 4-AP produced by the rapid pulsing, both the P1 I_{to} and the P2 tail currents should increase to final levels, therefore allowing analysis of tail current kinetics both under control conditions and in the presence of a fixed concentration of 4-AP but under varying degrees of block of activated I_{to} .

Although the tail currents generated by this protocol should be multiexponential (due to the fact that they were generated in the potential range where the activation variable, a , is not zero; see Campbell et al., 1993), in many myocytes they could be reasonably approximated by a single exponential. Fig. 9 shows representative results from one such myocyte where tail currents were first elicited in control and then in 0.5 mM 4-AP using the rapid pulse protocol (frequency = 1.25 Hz, total of 24 pulses). In the presence of 4-AP, rapid pulsing to +70 mV caused the amplitudes of both the P1 I_{to} (off scale in the figure) and the P2 tail currents (0 mV) to progressively increase. Approximating the tail currents as single exponentials, the time constants of deactivation in the presence of 0.5 mM 4-AP remained essentially constant during tail regrowth and were not different from control. Similar results (0.5 or 1 mM 4-AP) were obtained in a total of five myocytes. In none of the myocytes where the effects of 4-AP on tail currents were studied did we ever observe a crossover of the tail currents before and after application of 4-AP (i.e., similar to that observed for open channel block of the delayed rectifier K^+ current by quinidine: Colatsky et al., 1990; Snyders et al., 1991). The constancy of the (approximate) tail current time constants during the block/unblock cycle suggests that 4-AP does not significantly affect the open-to-closed state transition of deactivating I_{to} channels. However, while modification of

deactivating tail current kinetics is a strong supporting argument for open channel block, its absence is not necessarily an absolute argument against open channel block.

It is possible that rapid flickering block of the cardiac I_{to} channel might not affect the time course of deactivation if the channel could close normally with the blocking compound still bound within the channel pore binding site. However, we did not find any compelling evidence for trapping of 4-AP within the channel pore once the channel had been activated (cf. Choquet and Korn, 1992). When myocytes were held at HP = 0 mV and the tonic block protocol was applied (Fig. 3), the I_{to} elicited during both the first pulse to +50 mV and a second pulse applied 2 min later were the same.

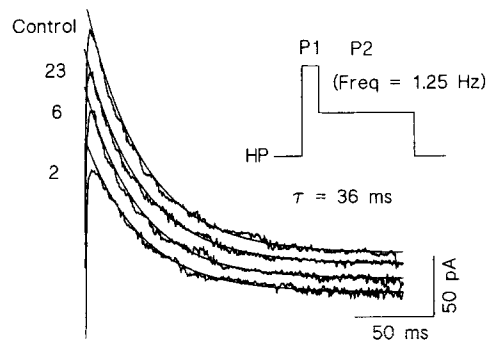


FIGURE 9. Lack of effect of 4-AP on the kinetics of deactivation of I_{to} tail currents. The voltage clamp protocol schematically illustrated in the inset (P1 = +70 mV, 15 ms; P2 = 0 mV, 150 ms) was rapidly applied at a frequency of 1.25 Hz in the presence of 0.5 mM 4-AP for a total of 24 pulses. Due to dissociation of 4-AP at +70 mV (see Fig. 7) the tail currents elicited during P2 progressively increased during the pulse train. Both a control tail current and tail currents for the

second, sixth, and next to last pulse of the train (denoted 2, 6, and 23) are illustrated. Tail current recordings have been offset from one another for clarity of presentation. Note that the time constants of deactivation (approximated as single exponentials; see text) were not altered ($\tau = 36$ ms) even though the degree of block by 4-AP was progressively relieved during the pulse train. The constancy of the tail current time constants suggests that 4-AP did not significantly alter the open-to-closed transition.

DISCUSSION

Our data demonstrate that 4-AP blocks I_{to} in ferret right ventricular myocytes. However, block at depolarized potentials is incomplete, even for concentrations of 4-AP up to 10 mM. Furthermore, the mechanism of block is complex, in that 4-AP both alters the kinetics of I_{to} (crossover phenomenon) and displays prominent voltage- and frequency-dependent characteristics (use dependence). In particular, the degree of block is lessened with either increasing frequency or depolarization. Therefore, the blocking actions of 4-AP on I_{to} in ferret right ventricular myocytes can be described as displaying reverse use dependence (Hondegheem and Snyders, 1990; Snyders, Hondegheem, and Bennett, 1991; cf. Hille, 1977, 1992; Hondegheem and Katzung, 1984).

Comparison with Use-dependent Effects of 4-AP Block of K^+ Channels in Other Cell Types

Potential- and frequency-dependent blocking effects of 4-AP have been previously observed for delayed rectifier-type K^+ currents of the squid axon (Yeh, Oxford, Wu,

and Narahashi, 1976; Kirsch and Narahashi, 1983), frog node of Ranvier (Ulbricht and Wagner, 1976), molluscan neurons (Hermann and Gorman, 1981), and rabbit Schwann cells (Howe and Ritchie, 1991). In many noncardiac cell types, block of inactivating I_{to} -like K^+ currents by 4-AP also displays use-dependent characteristics, with block frequently being relieved by depolarization. However, both the degree of, and the mechanisms underlying, such use-dependent block vary widely and depend on the specific type of I_{to} -like channel studied. For example, in GH₃ pituitary cells a relatively simple form of use-dependent block of I_{Kf} has been described, with 4-AP producing an initial transient open channel block followed by an equilibrium reduction of current that does not display any further voltage or frequency dependence (Wagoner and Oxford, 1990). In cloned *Drosophila Shaker* H4 channels expressed in *Xenopus* oocytes, block by 4-AP during depolarizing pulses is biphasic in character (an initial acceleration of the rate of apparent inactivation followed by a secondary increase of the current), suggesting rapid open channel block followed by slower unblock (Hice et al., 1992). In mouse lipopolysaccharide-activated B lymphocytes, Choquet and Korn (1992) have suggested that upon depolarization 4-AP becomes trapped within lymphocytic K^+ channels as they either subsequently inactivate or deactivate upon hyperpolarization (cf. Wagoner and Oxford, 1990). In contrast, no evidence could be found for either rapid open channel block or 4-AP binding to the inactivated state of I_{Kf} channels in rat melanotrophs (Kehl, 1990). Finally, an intermediate case appears to exist in molluscan neurons, where use-dependent block of I_A by 4-AP has been proposed to occur through both closed state binding and rapid open state binding followed by slower unbinding at depolarized potentials (Thompson, 1982). A similar mechanism also appears to apply to 4-AP block of I_A in guinea pig hippocampal neurons (Numann, Wadmann, and Wong, 1987). Our results are therefore most similar to those reported for 4-AP block of I_{Kf} in rat melanotrophs (Kehl, 1990), while they differ markedly from results obtained in *Drosophila* (Hice et al., 1992), GH₃ cells (Wagoner and Oxford, 1990), and mouse lymphocytes (Choquet and Korn, 1992). In summary, the mechanism of block of an I_{to} -like K^+ current by 4-AP in one cell type may not be applicable to I_{to} in another cell type.

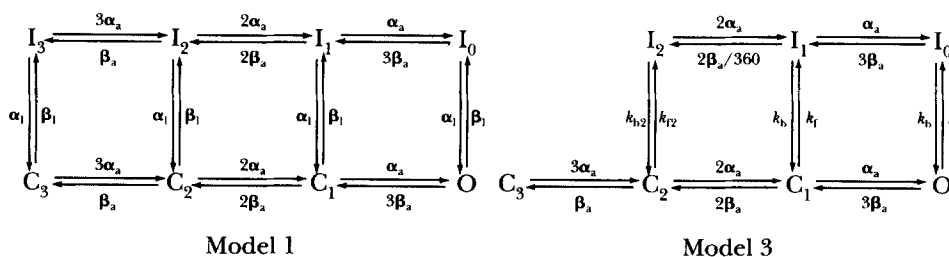
Quantitative Modeling and Reconstruction of Closed State Block of Ventricular I_{to} by 4-AP

One common form of state-dependent block of ion channels is pure open channel block. Use-dependent block in this case can arise when channel opening provides access to the binding site. If the channel cannot close or inactivate while the blocker occupies its binding site, then open channel block can produce crossover. However, as noted in Results, crossover of the deactivating tail currents in the presence of 4-AP was not observed (Fig. 9). Additionally, pure open channel block cannot reproduce development of tonic block at hyperpolarized potentials (Fig. 3) and relief of block at depolarized potentials (Figs. 5 and 7).

More complex models of open channel interactions with 4-AP-sensitive I_{to} -like K^+ channels have been postulated. For example, Choquet and Korn (1992) have proposed that 4-AP binds to, and blocks, the open state of voltage-gated K^+ channels in mouse lymphocytes. The blocked channel can deactivate and close, trapping 4-AP within the permeation pathway. Such a channel trapping model is unlikely to be

appropriate for characterization of the action of 4-AP on ferret ventricular I_{to} . Specifically, in mouse lymphocytes block does not occur at resting membrane potentials but only occurs after membrane depolarization and channel activation, while in ferret ventricular I_{to} 4-AP produces tonic block at resting membrane potentials (Fig. 3A). In addition, tonic block of ferret ventricular I_{to} could be prevented from occurring by holding the membrane potential at 0 mV (Fig. 3B), which produces <3% channel opening but nearly complete (92%) inactivation (Campbell et al., 1993). Thus, 4-AP binding and inactivation appear to be mutually exclusive.

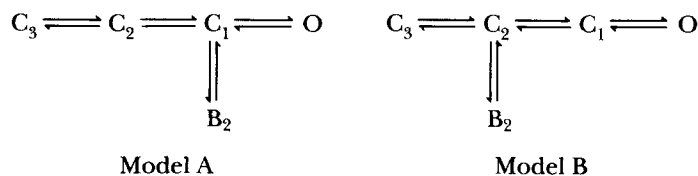
A detailed model of 4-AP binding to the ferret ventricular I_{to} channel should consider the putative mechanisms for inactivation described in the preceding paper (model 1 and model 3 in Campbell et al., 1993):

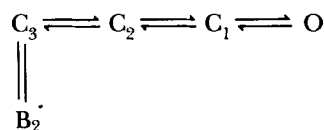


Both of these models have more than one inactivated state. The prevention of tonic block at 0 mV indicates that no substantial binding occurs to states I_1 and I_2 in model 1 or to state I_1 in model 3. Relief of block at +50 mV argues against any strong interactions of 4-AP with I_0 in models 1 and 3. I_3 in model 1 and I_2 in model 3 are only transiently occupied during return to the resting potential from depolarized potentials. Our measurements (Fig. 8) indicate that recovery from inactivation is unperturbed by 4-AP. 4-AP therefore does not interact significantly with the inactivated states during recovery, regardless of whether model 1 or 3 is chosen. We cannot rule out the possibility that 4-AP may have some low affinity for the inactivated states or may display binding that is very slow relative to the duration of occupancy of some inactivated states during recovery. For the purpose of modeling we have neglected the relatively small contribution of any such binding.

Models 1 and 3 differ only with respect to inactivation. Therefore, for the remainder of the discussion we show model simulations of binding to the closed states in the activation sequence (C_3, C_2, C_1, O) of model 3. Simulations using model 1 were also performed; the results were very similar and led to the same general conclusions as model 3. For convenience, model schematics will omit the inactivation mechanism.

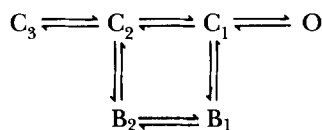
The simplest closed state binding model would entail 4-AP binding to only one closed state, giving three possible permutations:





Model C

where B_n denotes a 4-AP-bound closed state. Because the equilibrium between the various closed states in the activation sequence changes with membrane potential over the range -90 to -20 mV, models A and B predict that the affinity of the channel for 4-AP will be highly voltage dependent over this range, which is inconsistent with the voltage insensitivity measured in Fig. 6. In model C the relative occupancy of C_3 changes from 100 to 83% over the range -90 to -20 mV. Thus, the voltage dependence of block may be within the limitations of our experimental measurements. However, model C could not produce the time constant of dissociation at $+50$ mV without assuming a very complicated voltage dependence of binding. Similar arguments based on the voltage independence of the apparent K_d (Fig. 6) may be made against the following model incorporating binding to only two closed states:

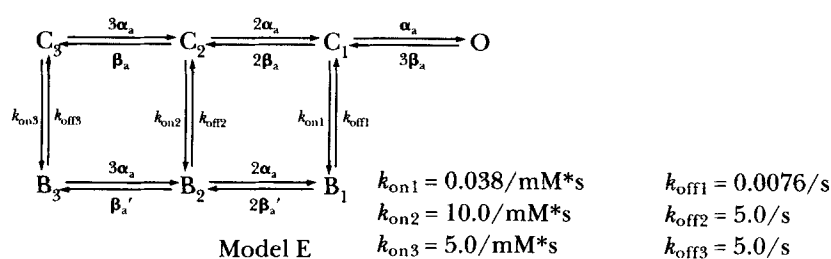


Model D

These results indicate that a more complex multiple closed state binding model must be considered. Because such models are complex, our ability to distinguish between various alternative formulations is diminished and must rely heavily on the ability of a particular binding model to reproduce the results quantitatively. This approach can be useful in developing a most likely model, but the results must be viewed within the limitations of our measurements. In particular, we have made the initial assumption that the quantitative description of the channel activation sequence (i.e., assignment of rate constants) is correct when considering binding of 4-AP to the channel. Because this assumption involves quantitative allocation of values to parameters that have not been measured directly (i.e., via gating current measurements: Bezanilla, Perozo, Papazian, and Stefani, 1992), alternative models cannot be rejected solely on the basis of quantitative inconsistencies. Because of this degree of uncertainty, our approach will be to present a single model that reproduces all of our data concerning the action of 4-AP on I_{to} . We wish to emphasize that the model to be presented represents only one of several potentially viable combinations of transitions and rate constants. The ambiguities associated with the assignment of rate constants to specific transitions between closed states are well known (Hille, 1992). Ultimately, these issues must be addressed by the direct measurements of the effects of 4-AP on gating currents and correlation with structure–function studies.

Multiple Closed State Binding Model

Reproduction of the time courses of both 4-AP association and dissociation requires that association occurs at a slower rate than dissociation (Figs. 5 and 7). The binding of 4-AP to the closed state of the channel from -90 to -20 mV produces a reduction in peak current that is voltage insensitive (Fig. 6). Over this range of potentials the ratio of $C_3:C_2$ changes from 1:0 to 83:16. On the basis of this observation we have applied the modeling constraint that the K_d values for 4-AP binding to the closed states of the channel are voltage insensitive. The K_d values may vary between states, resulting in an apparent voltage dependence, but binding and unbinding are assumed to be inherently voltage insensitive in the following modification of model 3:



The rate constants $\alpha(V)$ and $\beta(V)$ are given in the previous paper (Campbell et al., 1993). Because of the differing affinities for 4-AP, transitions between bound states were modified by assuming that α and β were decreased and increased proportionately to maintain detailed microscopic reversibility (Hille, 1992). These parameter values were initially estimated from the measurements presented in Results. However, the relationship between measurements and model parameters is not entirely straightforward when viewed quantitatively. This arises from the simplifying assumptions required for both experimental design and data analysis. For example, we propose that association is composed of binding to three closed states and is thus not a first-order process. Parameter values were therefore adjusted by simulating experimental protocols and analysis methods until results that were within 1 SD of experimentally observed values for τ_{assoc} , τ_{dissoc} , and apparent K_d were obtained.

The model successfully reproduces both the reduction in peak height of I_{to} and the general crossover phenomenon (Fig. 10 A). Fig. 10 B illustrates the model predicted concentration dependence of the reduction in peak I_{to} at $+50$ mV over the entire 4-AP concentration range used to estimate experimentally the apparent K_d (Fig. 2 B). The apparent K_d predicted by the model for block of peak I_{to} is 0.2 mM, which compares well with both the experimentally determined apparent K_d at $+50$ mV (0.2 mM) and the overall mean apparent K_d ($+20$ to $+100$ mV) of 0.19 ± 0.01 mM (Fig. 2 B).

When compared with the experimental data obtained in 10 mM 4-AP, the model successfully reproduces the phenomenon of voltage-dependent relief of block (Fig. 10 C). Both the potential of half-maximal relative block $V_{1/2}$ and its dependence on 4-AP concentration (Fig. 4 B) are adequately reproduced (10 mM 4-AP: model, $V_{1/2} = +18$ mV; measured, $V_{1/2} = +15$ mV; 1 mM 4-AP: model, $V_{1/2} = +2$ mV;

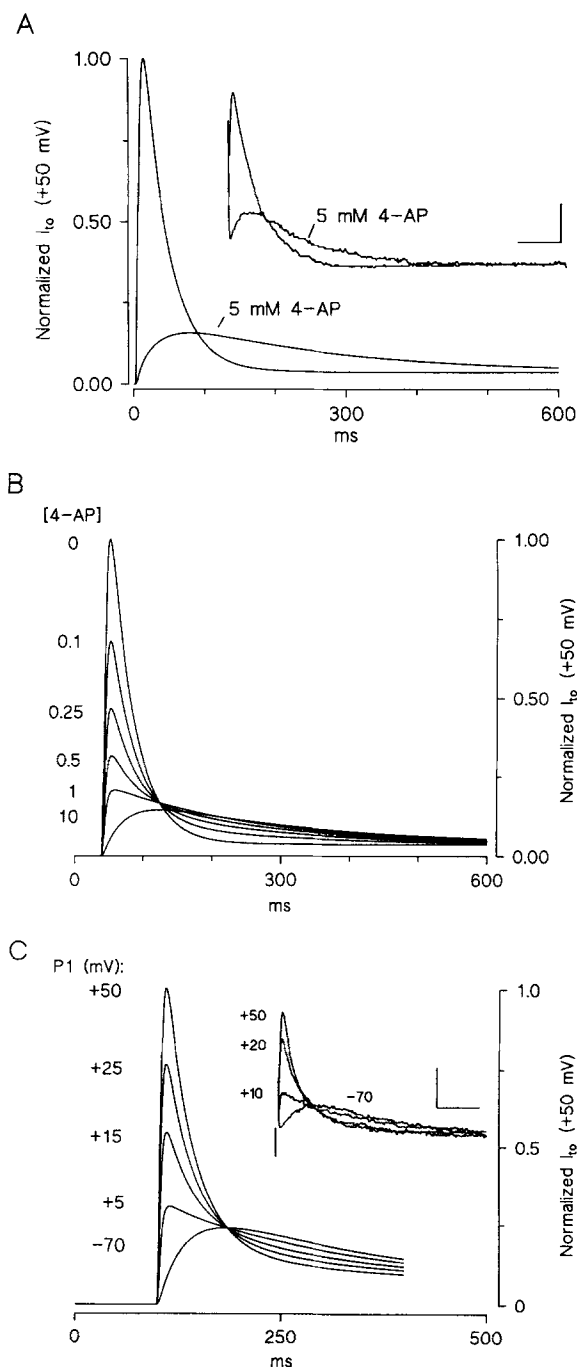


FIGURE 10. (A) Model-generated crossover behavior in 5 mM 4-AP. The main figure illustrates model-generated currents in control saline and 5 mM 4-AP at +50 mV (HP = -70 mV). The inset shows experimentally measured crossover behavior previously illustrated in Fig. 1. Inset calibration: 100 ms, 100 pA. (B) Model-predicted concentration dependence for the reduction in peak I_{to} at +50 mV (HP = -70 mV) over the [4-AP] range experimentally measured. The model predicted an apparent K_d for block of peak I_{to} of $K_{d,model} = 0.2$ mM (cf. Fig. 2). (C) Potential dependence of the relief of 4-AP block predicted by the model using the simulated double pulse P1-P2 protocol illustrated in Fig. 4 B. The main body of the figure shows model-generated I_{to} waveforms generated during the 500-ms P2 at +50 mV after a 500-ms P1 to the indicated potentials and a gap of 100 ms back to -70 mV. The inset shows the previous data presented in Fig. 4 A for comparison. Inset calibration: 100 ms, 200 pA.

measured, $V_{1/2} = +7$ mV). As can also be noted in Fig. 10, the crossover phenomenon was also reproduced, including the observation that crossover during the P2 pulse occurs at the same point in time, regardless of the level of the relief of block produced during the P1 pulse.

The simulated time dependence of association of 10 mM 4-AP at a holding potential of -70 mV is illustrated in Fig. 11 A. The model successfully reproduces the overall association behavior observed experimentally. The model-predicted approxi-

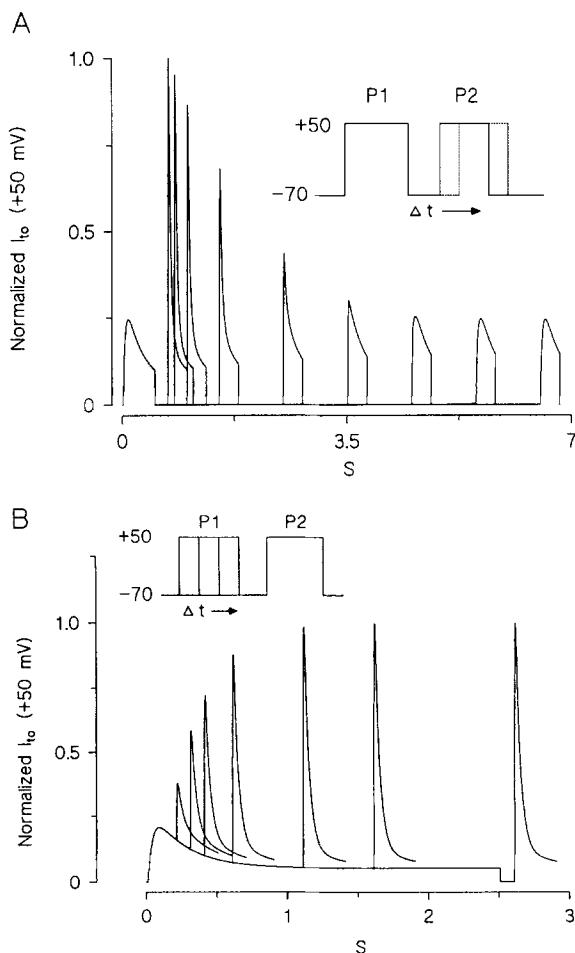


FIGURE 11. (A) Simulated time dependence of kinetics of 10 mM 4-AP association with the closed states of the I_{to} channel at HP = -70 mV. Simulated voltage clamp protocol is illustrated in the inset. The model generated a time-dependent 4-AP association that would be difficult to distinguish from a true exponential under experimental recording conditions and in the presence of noise. When the simulation results were analyzed as a single exponential process, an approximate time constant of 4-AP association at -70 mV of $\tau_{\text{assoc,model}} \approx 1$ s was obtained (cf. Fig. 5). (B) Model-generated time dependence of 4-AP dissociation kinetics at P1 = $+50$ mV in 10 mM 4-AP. Simulated voltage clamp protocol is shown schematically in inset. The model generated a time constant of 4-AP dissociation at $+50$ mV of $\tau_{\text{dissoc,model}} = 226$ ms (cf. Fig. 7).

mate time constant of 4-AP association at -70 mV is 1 s, a value within the range of experimentally measured association time constants (Fig. 5 C). The time dependence of 4-AP dissociation at $+50$ mV is simulated in Fig. 11 B using the identical double pulse P1-P2 protocol described in Fig. 7 B. The model successfully reproduces the observed dissociation phenomenon (Fig. 7 A). The model-predicted time constant of dissociation at $+50$ mV is 226 ms, a value again within the range of experimentally observed dissociation time constants (Fig. 7 C).

The multiple closed state binding model (modified model 3) is therefore able to successfully and quantitatively reproduce, within measured ranges of experimental variability, the five major phenomena that we have observed for block of I_{to} by 4-AP; i.e., (a) crossover and its kinetics, (b) the approximate dose–response relationship and apparent K_d for block of peak I_{to} , (c) association at hyperpolarized potentials, (d) dissociation at depolarized potentials, and (e) voltage dependence ($V_{1/2}$) of relative block.

Physical Interpretations

Under our recording conditions, >98% of 4-AP exists in the positively charged form 4-AP⁺ (see Methods). It is possible that the ~2% neutral form of the compound could permeate the lipid membrane and equilibrate in the intracellular space (e.g., Choquet and Korn, 1992). Whether the location of the 4-AP binding site(s) on the ferret ventricular I_{to} channel is extracellular, intracellular, or intramembranous (see Howe and Ritchie, 1991) remains undetermined. While we obtained no evidence for open channel block, we cannot rule out the possibility that 4-AP binds at or near the mouth of the pore. However, any such binding does not experience a substantial portion of the transmembrane electric field (Fig. 6).

The rate of binding and unbinding of 4-AP to the closed state of the I_{to} channel is very slow compared with the rates of binding and unbinding of other K⁺ channel blockers. For example, quinidine binds and unbinds to the open state of the cloned human delayed rectifier K⁺ channel (HK2) with kinetics that are approximately three orders of magnitude faster (Snyders et al., 1992). Furthermore, the rate of binding and unbinding of 4-AP in the hyperpolarized range was voltage dependent, while the relative fractional block of the current over this same range indicated that the apparent K_d remained constant (Fig. 6). One particularly attractive explanation for this observation is that binding and unbinding of 4-AP is restricted by a portion of the I_{to} channel protein. The nature of this postulated steric hindrance is unclear at the moment. To reproduce our results, both binding and unbinding must be hindered in an approximately proportional fashion. The kinetic differences associated with this effect have been modeled by different rate constants associated with each of the individual states in the activation sequence. It is possible that the observed voltage dependence of the kinetics of association is the result of direct coupling to the channel conformational changes associated with activation. However, the postulated steric hindrance may exhibit an intrinsic voltage dependence that is independent of the activation process.

Both the data and the model suggest that 4-AP binding and inactivation are mutually exclusive processes. Because 4-AP binding and steady-state inactivation can occur at overlapping potentials (Fig. 6), one would expect interactions between inactivation and 4-AP binding. Such interactions are also suggested by the observation that voltage-dependent relief of block by 4-AP at low concentrations roughly parallels steady-state inactivation, while at higher concentrations relief of block occurs at more depolarized voltages (Fig. 4). It is most likely that the interaction is allosteric; i.e., 4-AP may stabilize a channel conformational state that displays a low affinity for the inactivation gating particle, or delays transitions to inactivatable states.

Possible Pharmacological, Clinical, and Molecular Implications for Block of Cardiac K⁺ Channels

Many so-called class III antiarrhythmic drugs (e.g., amiodarone, sotalol, clofilium) prolong action potential duration by blocking K⁺ channels (e.g., Colatsky, 1990). Recent hypotheses (see Hondeghem and Snyders, 1990) have suggested that some of these class III compounds exert their effects on cardiac K⁺ channels through a reverse use-dependent mechanism. Specifically, these drugs are hypothesized (a) to bind to the closed state of K⁺ channels and (b) to either have a low affinity for or dissociate from the open state. Therefore, block will increase during diastole and decrease during the plateau, giving less block with increased frequency. As a result, such drugs are more effective at reduced heart rates. The hypothesized characteristics of such class III antiarrhythmics are therefore remarkably similar to those we have measured and quantitatively modeled for 4-AP block of I_{to} . Our model of closed state block of I_{to} by 4-AP may serve as a useful general model for understanding block of cardiac K⁺ channels by certain therapeutic compounds. It will be very interesting to see how future kinetic studies on the effects of such class III compounds on I_{to} compare with our 4-AP analysis.

Finally, at the molecular level the differences between the mechanisms of 4-AP block may serve as a useful pharmacological tool for further classification of different I_{to} channel types (see Rudy, 1988). 4-AP and similar compounds have the potential to become powerful tools for gaining insight into the molecular mechanisms governing cardiac K⁺ channel gating (e.g., Miller, 1991; Jan and Jan, 1992). By exclusively binding to closed states, the use of 4-AP or similar compounds could potentially allow for testing of specific models of cardiac K⁺ channel gating, give insights into mechanisms underlying cardiac K⁺ channel neuromodulation, and serve as a probe of the underlying molecular mechanisms governing transitions among the various closed states.

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