Voltage-dependent Modulation of Ca Channel Current in Heart Cells by Bay K8644

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ABSTRACT We have investigated the voltage-dependent effects of the dihydropyridine Bay K8644 on Ca channel currents in calf Purkinje fibers and enzymatically dispersed rat ventricular myocytes. Bay K8644 increases the apparent rate of inactivation of these currents, measured during depolarizing voltage pulses, and shifts both channel activation and inactivation in the hyperpolarizing direction. Consequently, currents measured after hyperpolarizing conditioning pulses are larger in the presence of drug compared with control conditions, but are smaller than control if they are measured after positive conditioning pulses. Most of our experimental observations on macroscopic currents can be explained by a single drug-induced change in one rate constant of a simple kinetic model. The rate constant change is consistent with results obtained by others with single channel recordings.

INTRODUCTION

The dihydropyridine Ca channel blockers such as nifedipine, nisoldipine, and nitrendipine are the most specific and potent of all the Ca channel antagonists that have been investigated (reviewed by Janis and Triggle, 1983, 1984). In addition to these drugs, there exists a series of structurally similar compounds, the Ca channel agonists, whose primary action is the enhancement of Ca-dependent processes in a number of cells. Single channel studies have revealed that these drugs, typified by Bay K8644 and CGP 28 392, increase macroscopic currents by prolonging the mean open time of the channel (Hess et al., 1984; Ochi et al., 1984; Kokubun and Reuter, 1984). Such effects on Ca channel currents probably underlie the drugs' positive inotropic actions (Schramm et al., 1983*a*; Thomas et al., 1984), smooth muscle stimulation (Schramm et al., 1983*b*; Su et al., 1984), and enhancement of neurotransmitter secretion by isolated cells in culture (Freedman and Miller, 1984; Enyeart and Hinkle, 1984; Garcia et al., 1984).

We have previously reported (Sanguinetti and Kass, 1984*b*) that a constant

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concentration of Bay K8644, in addition to acting as a Ca channel agonist, can decrease Ca channel currents and contractile activity, depending on membrane potential. At relatively hyperpolarized holding potentials, Bay K8644 enhances peak inward current, measured during a depolarizing test pulse, but decreases Ca channel current if the holding potential is depolarized. Thus, membrane potential determines whether Bay K8644 acts as a Ca channel antagonist or agonist. High concentrations of Bay K8644 (30 μM) have also been reported to depress the contractility of isolated hearts (Thomas et al., 1984) and to attenuate the enhancement of contractility of isolated coronary artery rings observed with lower drug concentrations (Vaghy et al., 1985). Furthermore, experiments with rat tail arteries have shown that inhibitory actions of Bay K8644 are most pronounced when these cells are depolarized by high concentrations of extracellular K (Su et al., 1984).

The nature of the antagonism of Ca channel currents by Bay K8644 has not been systematically investigated, although it has been suggested that this drug acts as a partial agonist, causing inhibition of Ca channel currents by a mechanism similar to nisoldipine and other dihydropyridine antagonists (Hess et al., 1984).

The purpose of this study was to investigate more completely the voltage-dependent actions of Bay K8644. We find that most of the effects of this drug, including enhancement and inhibition, can be explained by a change in channel gating consistent with that reported in single channel studies.

A preliminary report of these results was presented to the Biophysical Society (Sanguinetti and Kass, 1985).

METHODS

These experiments were carried out in isolated calf Purkinje fibers and in single myocytes isolated from rat ventricle using an enzymatic dissociation procedure modified from Farmer et al. (1983). Methods for Purkinje fiber experiments are as follows. The two-microelectrode technique used to measure membrane currents and the standard Tyrode's solution used in this study are described elsewhere (Kass and Sanguinetti, 1984). Experiments were carried out at 37°C and solutions were gassed with 100% O_2. Outward currents were blocked by the injection of tetrabutylammonium (Kass et al., 1982) and Na currents were blocked by 10 μM tetrodotoxin (TTX). In most experiments, Ca was replaced by Sr to promote larger currents through Ca channels (Hess and Tsien, 1984) and to minimize the contribution of Ca-dependent inactivation (Kass and Sanguinetti, 1984).

Single cell experiments were carried out at 20°C and the standard external solution consisted of (mM): 132 NaCl, 4.8 CsCl, 10 HEPES, pH 7.3, 5 glucose, 1.2 $MgCl_2$, and 1 $CaCl_2$. Na channel currents were blocked by TTX (10–50 μM). The patch pipette solutions consisted of (mM): 140 CsCl, 2 $MgCl_2$, 11 EGTA, 1 $CaCl_2$, and 10 HEPES, pH 7.3. The resistances of the fire-polished electrodes ranged from 2.5 to 3.5 MΩ when this pipette solution was used. Membrane currents were measured with a patch pipette arranged in a whole-cell configuration (Hamill et al., 1981). The presence of EGTA in the patch electrode helps minimize Ca-dependent inactivation, and CsCl was used to block the transient outward current in the single cells. In each single cell experiment, pipette capacitance was neutralized after a seal was formed. Series resistance was compensated after the seal was broken, to provide the fastest possible capacity transient without ringing.

Curve-Fitting Procedures

Boltzmann functions were used to describe Ca channel activation and inactivation relationships. Theoretical curves were fitted to experimental data using an algorithm of Marquardt (1963) as described by Bevington (1969). This procedure, which fits an arbitrary nonlinear function to the data, was thus used to determine $V_{½}$ and k for appropriate Boltzmann functions and to determine the best-fitting exponential function plus baseline to describe the time course of the data.

Measurement of Inactivation and Activation

Ca channel current availability was determined by measuring current during test pulses to voltages near the peak of the Ca channel current-voltage relation after applying prepulses to several conditioning potentials. Prepulse durations were chosen for particular experiments. Test pulses were separated from prepulses by a 10-ms return to the holding potential. Curves were normalized to currents measured after the most negative prepulses.

For Purkinje fiber experiments, activation curves were determined by measuring background (I_B) and capacitance currents in the presence of a high nisoldipine concentration (10 μM) and subtracting these from control and Bay K8644 records. After subtraction of background currents, the time course of inactivation was determined by the best-fitting function with two exponential components, and the amplitude of the inward current at the start of the test voltage pulse was determined by extrapolation (shown in the inset of Fig. 4). This value was then plotted against the test voltage pulse in the absence and presence of Bay K8644. The Ca channel current reversal potential was then determined from the leak-subtracted data and the driving force was computed for each test voltage. The extrapolated current and driving force were used to determine conductance and, in turn, activation curves.

A similar procedure was followed for single cell experiments, but in this case I_B was extrapolated from current measured at V_{rev} by assuming a linear current-voltage relationship for I_B. This assumption is reasonable because the inclusion of CsCl in the pipette solution blocks nonlinear outward currents (Matsuda and Noma, 1984).

Drugs

Bay K8644 and nisoldipine were kindly supplied by Miles Laboratories, New Haven, CT. These drugs were dissolved in polyethelene glycol 400 (PEG 400) to make a concentrated stock solution (10 mM). Aliquots of stock solution were diluted into Tyrode's solutions to obtain the working concentrations. Control tests have shown that PEG 400 has no effects on Ca channel currents at concentrations 50 times higher than used in this study (Kass, 1982). TTX was obtained from Sigma Chemical Co., St. Louis, MO.

RESULTS

Multiple Actions of Bay K8644

Bay K8644 causes several changes in Ca channel current, which can be seen in Fig. 1. The figure shows the effects of exposure to several concentrations of Bay K8644 (40, 100, and 500 nM, and 2 μM) on membrane currents in a Purkinje fiber measured during pulses applied from a holding potential of −50 mV. The inset shows currents measured during pulses to −4 mV, and the peak inward currents measured at a series of voltages are plotted in the main part of the figure. Currents were measured 30 min after the addition of a given concentra-

tion of the drug. In each concentration of the drug, there was an increase in the magnitude of peak inward current measured at −4 mV (inset) and over most of the current-voltage relationship.

Two other effects are also apparent. In the presence of Bay K8644, the peak of the current-voltage relationship shifted in the hyperpolarizing direction and much of the shift appeared at low concentrations of the drug. Resolution of the

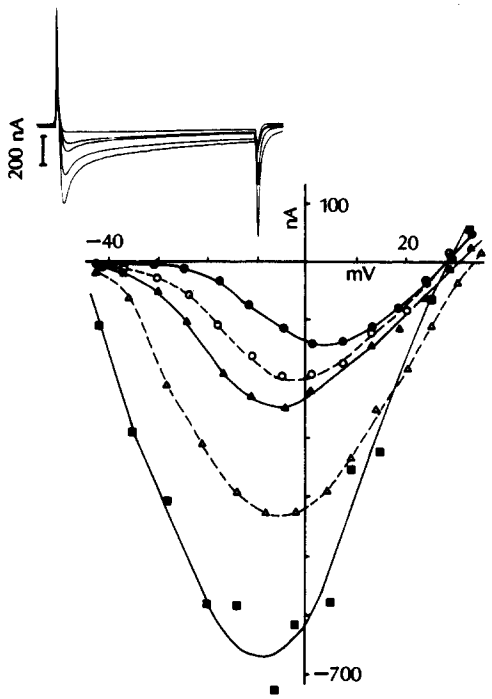


FIGURE 1. Concentration-dependent effects of Bay K8644 on Purkinje fiber net currents. Membrane currents were measured in control, drug-free conditions (●) and after exposure to 40 nM (○), 100 nM (▲), 500 nM (△), and 2 μM (■) Bay K8644. Drug concentrations were applied consecutively from the lowest to highest concentrations, and currents were measured 30 min after exposure to a given concentration. Currents were measured with continuous microelectrode impalements in a single preparation. Inset: currents in response to 100-ms voltage pulses to −4 mV in each solution. Curves show peak currents measured in response to pulses to the voltages indicated along the horizontal axis in each solution. The curves are hand-drawn. HP, −50 mV. 5.4 mM Sr. Fiber 359-2.

precise magnitude of this shift in each concentration of Bay K8644 in this type of experiment is difficult because of the scatter in the data and because of the broad voltage dependence of the currents near the peak of the current-voltage relationship. However, drug-induced shifts of comparable magnitude in the peak of the current-voltage relationship were observed during each application of the drug in both single cell and Purkinje fiber experiments. Table I provides a summary of these results.

In addition to the change in the *I-V*, the apparent rates of inactivation of currents shown in the inset of Fig. 1 were faster in the presence of the drug and this effect was also seen in single cell experiments (see the inset of Fig. 6). Fig. 2 shows that Bay K8644 sped the rate of inactivation of Ca channel currents, not just at −4 mV, but over a wide range of test voltages. Both components of the

TABLE I

Bay K8644: Shift in Peak of I-V

Purkinje fibers Experiment	Charge carrier	Concentration of Bay K8644	HP	Shift (*I-V*)
343-3	Ca	100 nM	−45	−10.0
343-4	Ca	200 nM	−42	−5.0
344-2	Ca	100 nM	−57	−5.0
344-2	Ca	500 nM	−57	−12.0
350-1	Ca	2 μM	−60	−15.0
351-1	Ca	500 nM	−62	−10.0
351-1	Ca	500 nM	−45	−10.0
352-1	Sr	500 nM	−54	−8.0
352-2	Sr	500 nM	−66	−8.0
352-2	Sr	500 nM	−45	−10.0
354-1	Sr	500 nM	−51	−8.0
354-2	Sr	500 nM	−69	−7.0
358-1	Sr	400 nM	−50	−10.0
384-1	Sr	500 nM	−58	−2.0
384-2	Sr	500 nM	−56	−5.0
353-1	Sr	500 nM	−56	−5.0
359-2	Sr	500 nM	−56	−9.0
(N = 14)		Mean shift (500 nM, N = 10)		
		−7.5 ± 0.92		

Single cells Cell	Charge carrier	Concentration of Bay K8644	HP	Shift (*I-V*)
0785BF, HI	Ca	500 nM	−50, −60	−8
0785BL, N-O	Ca	500 nM	−60	−10
1015B2	Sr	500 nM	−55	−6
101521	Sr	100 nM	−50	−15
101523	Sr	1,000 nM	−50	−16
101551	Sr	50 nM	−55	−5
10154	Sr	1,000 nM	−55	−15
10181	Sr	1,000 nM	−60	−10
10251	Sr	50 nM	−45	−7
10251	Sr	100 nM	−45	−20
(N = 10)				

biexponential decay of Ca channel currents (Kass and Sanguinetti, 1984) were affected by Bay K8644. The remainder of this study addresses the basis for these drug actions.

The change in kinetics of inactivation and the hyperpolarizing shift in the current-voltage relationship are effects that might be attributed to a loss of voltage control caused by the magnitude of the inward current measured in the

presence of the drug (see Brown et al., 1984). Thomas et al. (1984) provided evidence against such an artifact by using a reduced concentration of the permeant divalent cation. We tested for current-dependent artifacts in the Purkinje fiber by increasing the magnitude of the inward current with norepinephrine.

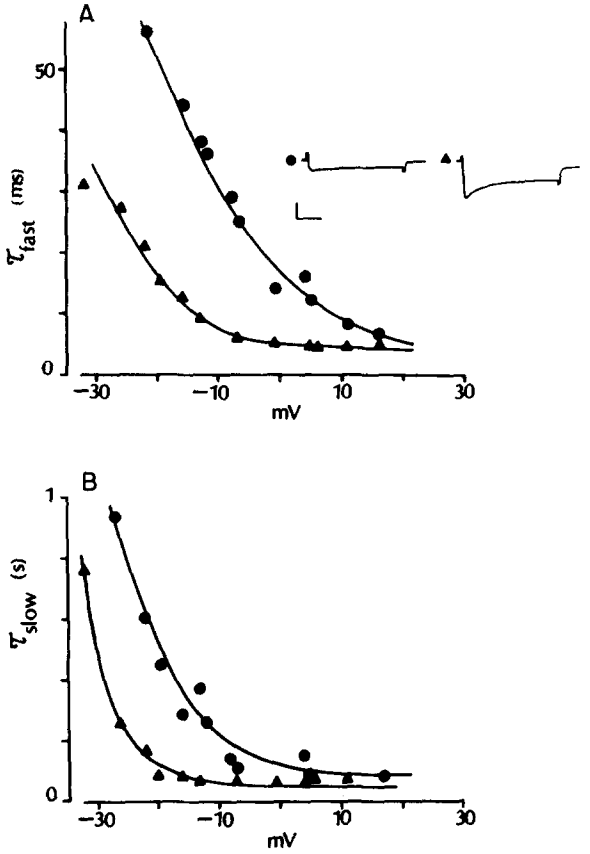


FIGURE 2. Bay K8644 reduces both time constants of inactivation in the Purkinje fiber. Current was measured during voltage pulses applied from a −56-mV holding potential and the time constants of inactivation were determined as described in the Methods. Both the fast (*A*) and slow (*B*) time constants were determined in the absence (●) and presence (▲) of 500 nM Bay K8644 and plotted against pulse voltage. Inset: currents measured at −13 mV in the absence and presence of drug. Capacity transients have been omitted to conserve space. The curves are hand-drawn. 5.4 mM Sr. Fiber 353-1.

Fig. 3 shows current-voltage relationships in a Purkinje fiber for peak inward current measured before and after exposure to 5 μM norepinephrine, a maximally effective concentration for enhancing Ca channel current in the calf Purkinje fiber (Kass and Wiegers, 1982). Peak currents were increased about threefold, but there was little effect on the position of the maximum in the

current-voltage relationship. In the continued presence of norepinephrine, the addition of Bay K8644 (0.4 μM) caused an additional enhancement of current and a 10-mV hyperpolarizing shift of the peak of the current-voltage relationship. The subsequent washout of the norepinephrine reduced the magnitude of the currents, but did not reverse the shift in the current-voltage relationship, which argues against an artifact induced by the current magnitude. (Note that the magnitude of the peak of the *I-V* in Bay K8644 alone is very similar to that in norepinephrine alone.) This experiment shows that, as previously demonstrated (Thomas et al., 1984), Bay K8644 and norepinephrine increase Ca channel currents by different mechanisms.

We also found that the shift in the peak of the current-voltage relationship

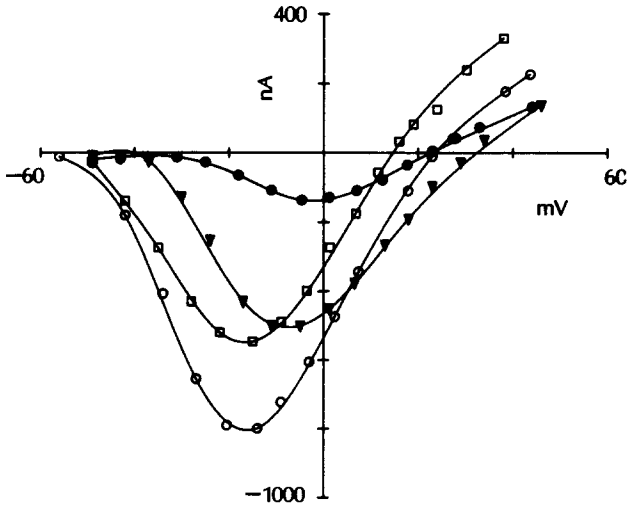


FIGURE 3. Test for current-dependent effects of Bay K8644 in the Purkinje fiber. Peak currents were measured in the following solutions and in the following order (see text for details): control (●), 5 μM norepinephrine (▼), norepinephrine plus Bay K8644 (○), and 0.4 μM Bay K8644 (□). The hand-drawn curves show peak current vs. voltage in each solution. Currents were measured with continuous impalements in a single preparation. 5.4 mM Sr. Fiber 358-1.

was not dependent on current amplitude in single cells. In these experiments, we applied Bay K8644 to cells held at potentials between −60 and −50 mV (Fig. 4). As was the case in the Purkinje fiber experiments, this caused an increase in current amplitude and a hyperpolarizing shift in the peak of the current-voltage relationship (Fig. 4, open squares). We remeasured the currents at different test voltages after applying brief (500 ms) conditioning pulses to −35 mV to inactivate some Ca channels and thus reduce current amplitude. The choice of the prepulse voltage allowed us to control the current amplitude in the presence of the drug and thus to provide a comparison of control and drug-altered currents of comparable magnitudes. The voltage-dependent shift was not affected by this procedure in any of four experiments in which it was carried out (see Fig. 4, open triangles).

Bay K8644 Shifts the Voltage Dependence of Ca Channel Current Activation

The current-voltage relationships shown in Figs. 1 and 3 include background as well as Ca channel currents. To test for actions of Bay K8644 on Ca channel current activation, background (or leak) currents must first be subtracted from total current records (see Methods).

The leak-subtracted current-voltage relationships presented in Fig. 5 indicate

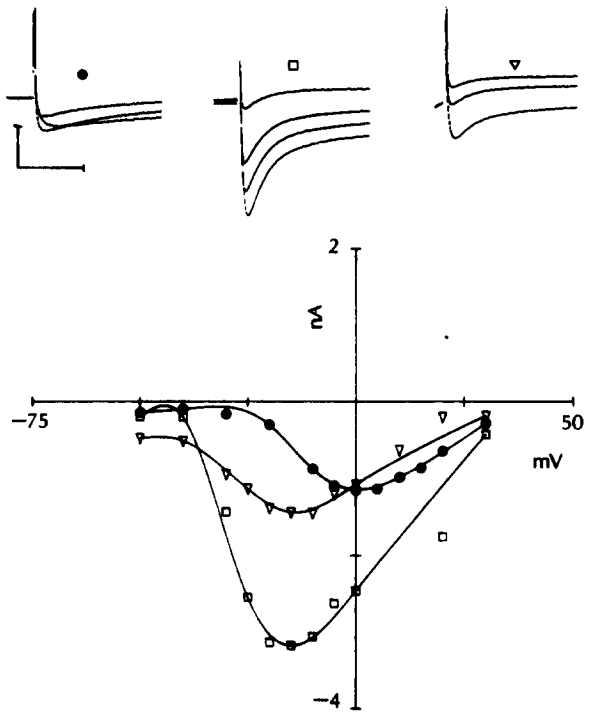


FIGURE 4. Bay K8644–induced voltage shift is not current dependent in the single cell. Peak currents were measured from a −55-mV holding potential in the absence (●) and presence (□) of Bay K8644 (500 nM) and in the presence of the drug but after applying 500-ms prepulses to −35 mV to inactivate some Ca channels (▽). The insets show currents recorded at −10, 0, and +20 mV in each condition (additional sweep in □ is to +30 mV). Curves are spline fits to the data. Calibration bars: 1 nA, 50 ms. Cell 1015-2.

a 10-mV hyperpolarizing shift in peak current in the presence of Bay K8644, but no change in the Ca channel current reversal potential (+53 mV) (also see Hess et al., 1984). Thus, the effect on the current-voltage relationship must be due to a hyperpolarizing shift in the activation curve. This can be seen in Fig. 6, which presents conductance vs. voltage relationships (determined as described in the Methods). The figure shows a fourfold increase in maximal conductance (*A*), and clearly indicates a 10-mV shift in this curve (*B*, normalized data). In each of two other Purkinje fiber experiments in which the shift in Ca channel activation

was measured, it was also found to be the same as the change in the peak of the current-voltage relationship (−6 and −9 mV for the two additional experiments).

Fig. 7 illustrates that Bay K8644, applied at a comparable concentration, also caused a hyperpolarizing shift in the Ca channel activation curve determined (as described in the Methods) in single cells.

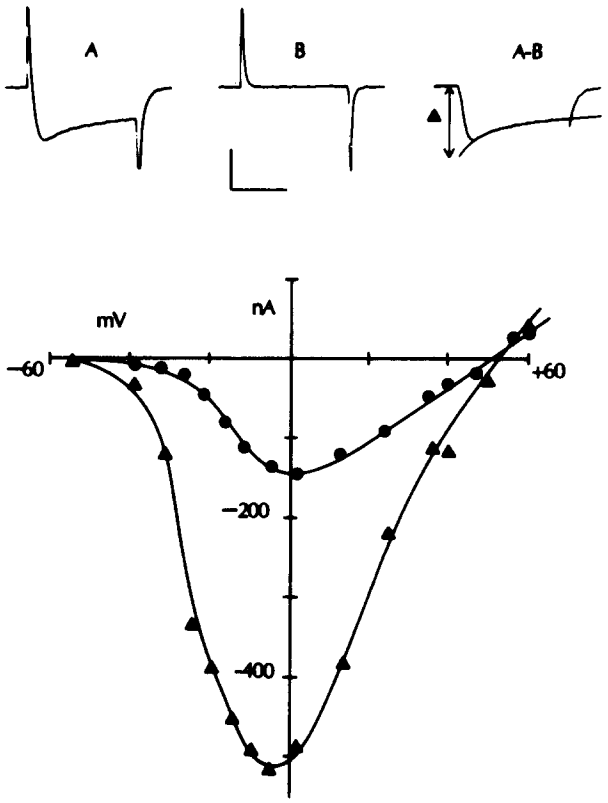


FIGURE 5. Influence of Bay K8644 on leak-subtracted Purkinje fiber currents. Inset: background currents measured in the presence of 10 μM nisoldipine (*B*) were subtracted from those in the presence of 500 nM Bay K8644 (*A*) to determine the effect of Bay K8644 on leak-subtracted records. Difference records (*A* − *B*) are fitted with functions with two exponential components (Methods). Curves: difference current at the start of the test pulse, determined by extrapolation, is plotted against pulse voltage in the absence (●) and presence (▲) of Bay K8644. The curves are hand-drawn. 5.4 mM Sr. Fiber 353-1.

Bay K8644 Shifts Inactivation Measured with Brief Prepulses

We previously showed (Sanguinetti and Kass, 1984*a*) that 200 nM nisoldipine causes an ~20-mV hyperpolarizing shift in the steady state inactivation relationship when conditioning prepulses are 30 s long, but does not affect the inactivation curve if prepulses are 500 ms in duration.

Bay K8644 affects the relationship between inactivation and voltage in a different manner. Substantial shifts in the inactivation curve can be measured after the application of very brief conditioning pulses. In addition, the dual actions of this drug cause a "crossover" of the inactivation relationship. Unnormalized data for 500-ms inactivation curves obtained in a Purkinje fiber in the absence and presence of Bay K8644 (200 nM) are presented in Fig. 8. There

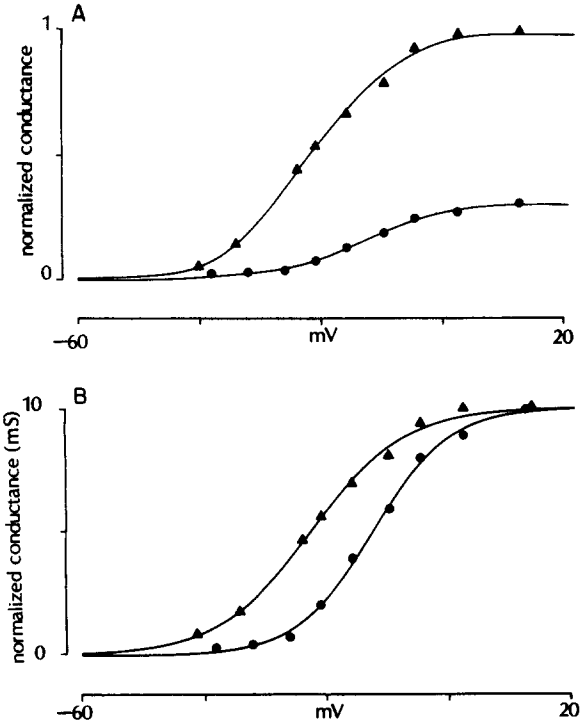


FIGURE 6. Influence of Bay K8644 on conductance and normalized conductance vs. voltage relationships in the Purkinje fiber. (*A*) Conductance was obtained as described in the Methods and plotted against test voltage. Bay K8644 (500 nM) increases the maximal conductance from 2.2 to 9 ms. (*B*) The data in *A* were normalized to the maximal conductance in the absence and presence of drug and plotted against test pulse voltage. The smooth curves are the best-fit Boltzmann relationship obtained as described in the Methods. Symbols for both panels: control (●), drug (▲). Parameters describing these curves are (in millivolts): $V_{1/2} = -12.6$, $k = 6.2$ (control); $V_{1/2} = -22.6$, $k = 7.6$ (drug). 5.4 mM Sr. Fiber 353-1.

was a roughly twofold increase in current measured after negative conditioning pulses, but a decrease in current measured after positive conditioning voltages (also seen in the inset).

These data are plotted as normalized curves in Fig. 9*A*. As can be seen from the normalized data, 200 nM Bay K8644 caused a 12-mV hyperpolarization of the relationship between inactivation and membrane potential measured with 500-ms conditioning pulses. Increasing the Bay K8644 concentration 50-fold

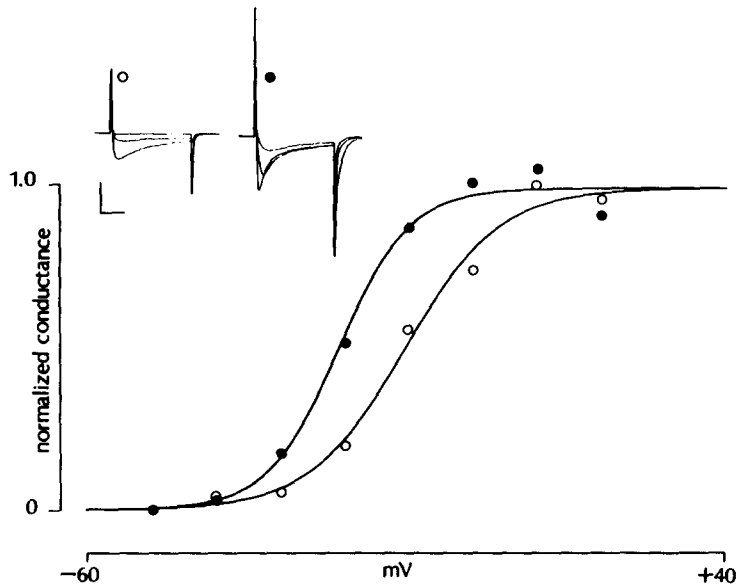


FIGURE 7. Normalized conductance vs. voltage curves from a rat ventricular cell in control (○) and in the presence of 500 nM Bay K8644 (●). The holding potential was −50 mV in control and −70 mV in drug. Conductance and the fitted curves were calculated as described in the Methods. $V_{½}$ and k were −10.4 and 7.3 in control and −20.6 and 5.5 in Bay K8644. The inset shows membrane current during test pulses to −30, −20, and 0 mV in control and drug. Calibration bars: 1.2 nA, 25 ms. Cell 0785BH; $[Ca^{2+}]_o$ = 1.0 mM.

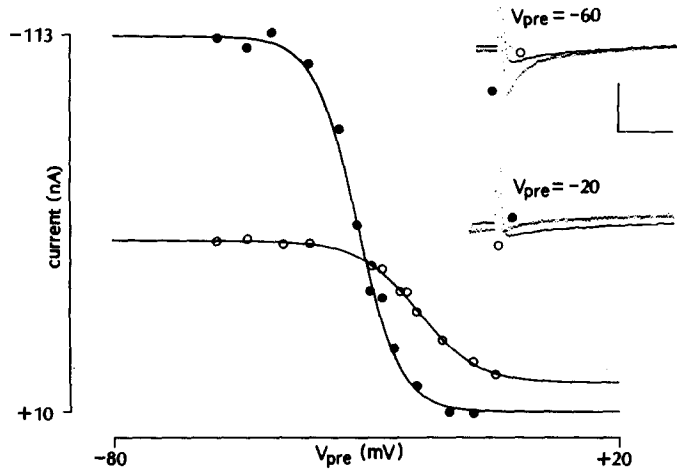


FIGURE 8. Bay K8644 shifts Ca channel availability in the Purkinje fiber. Ca channel availability was determined by measuring currents in response to test pulses to 0 mV after 500-ms steps to conditioning voltages. Note that there was a 10-ms break between conditioning and test pulses. The absolute value of peak current is plotted against conditioning voltage in the absence (○) and presence (●) of 200 nM Bay K8644. Curves are best-fit Boltzmann relationships to the data. Parameters: $V_{½}$ = −32 mV, k = 5.2 mV (Bay K8644); $V_{½}$ = −20 mV, k = 5.7 mV (control). Inset: currents measured at 0 mV after conditioning steps to −60 and −20 mV in the absence and presence of drug. 5.4 mM Sr. Fiber 386-2.

caused no further shift of this relationship. A prolongation of the conditioning pulse from 0.5 to 10 s promotes additional slow inactivation of Ca channels in the absence of Bay K8644 and thus causes a hyperpolarizing shift in the control inactivation curve (Kass and Scheuer, 1982; Sanguinetti and Kass, 1984*a*; Kass

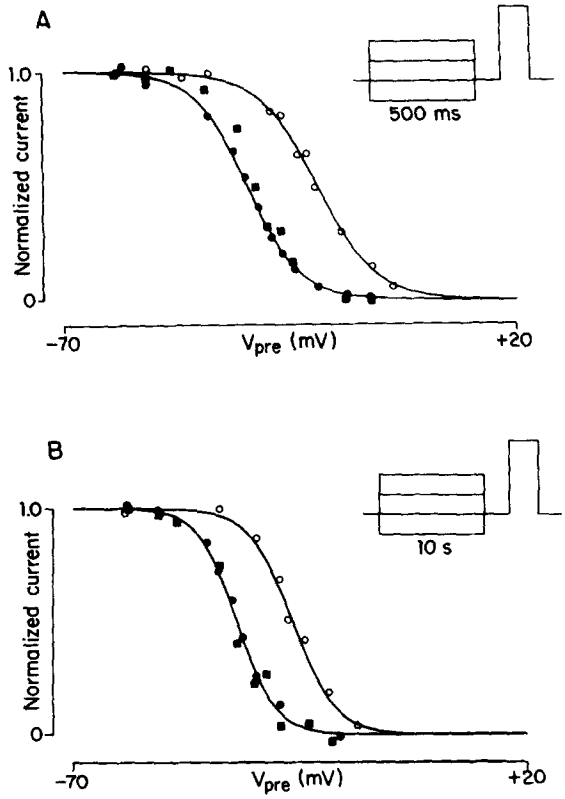


FIGURE 9. Influence of drug concentration and conditioning pulse duration on Bay K8644–induced shift of inactivation curve. Inactivation curves, determined as described for Fig. 7, were measured in control (○), 200 nM Bay K8644 (●), and 10 μM Bay K8644 (■). In each solution, the data were normalized to currents measured after the most negative conditioning voltages. These currents were 45, 130, and 165 nA in control and 200 nM and 10 μM Bay K8644. The holding potential was −55 mV, and the test voltage was 0 mV. The smooth curves are the best-fit Boltzmann relationships (Methods) in each solution. The parameters that follow are in millivolts. (*A*) Inactivation assayed with 500-ms conditioning pulses. $V_{½} = -20$, $k = 6$ (control); $V_{½} = -32$, $k = 4.1$ (200 nM); $V_{½} = -33.6$, $k = 5.1$ (10 μM). (*B*) Inactivation after 10-s conditioning pulses. $V_{½} = -26.6$, $k = 4.2$ (control); $V_{½} = -38.3$, $k = 3.9$ (200 nM); $V_{½} = -37.8$, $k = 3.7$ (10 μM). 5.4 mM Sr. Fiber 386-2.

and Sanguinetti, 1984). However, the increase in prepulse duration did not affect the Bay K8644–induced shift in inactivation (Fig. 9*B*). Thus, it is not likely that slow inactivation is important to this action of the drug.

We obtained similar results in single ventricular cells, as can be seen in Fig.

10. After negative conditioning pulses, Bay K8644 enhanced Ca channel currents but decreased current measured after positive prepulses (see inset). When plotted as normalized current, there is a clear hyperpolarizing shift in the relationship between Ca channel availability and prepulse voltage.

Table II provides a summary of all of our experiments testing for Bay K8644–induced shifts of Ca channel availability in single cells and Purkinje fibers.

Modulation of Current by Bay K8644 Is Frequency Dependent

Fig. 11 presents the results of an experiment designed to test for frequency-dependent actions of Bay K8644. Here the pulse frequency, not conditioning

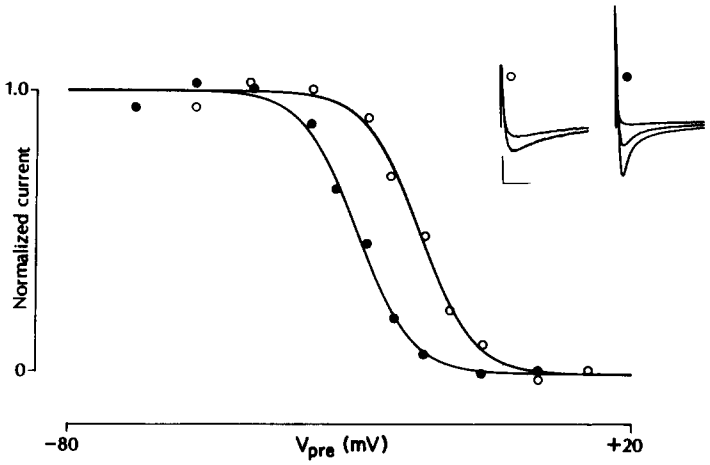


FIGURE 10. Shift of Ca channel availability by Bay K8644 in the single cell. Ca channel availability determined as described for Fig. 7, but in an enzymatically dispersed single ventricular cell in control (○) and drug (●). The conditioning pulse duration was 500 ms and the holding potential was −60 mV. Smooth curves are Boltzmann relationships fitted to the data as described in the Methods. Parameters (in millivolts): $V_{½}$ = −17.7, k = 4.6 (control); $V_{½}$ = −28.8, k = 4.7 (Bay K8644). Values of currents corresponding to normalized values of 1 are 500 pA (control) and 1,246 pA (Bay K8644). Inset: current during test pulse after prepulses to −60, −30, and −20 mV in the absence and presence of drug. Calibration bars: (control) 249 pA; (drug) 623 pA and 28 ms. The concentration of Bay K8644 was 500 nM; 50 μM TTX was used to block I_{Na}. Ca_o = 1 mM. Cell 0785A6.

(or holding) potential, was varied. The holding potential was −66 mV, and currents were measured during brief (50 ms) pulses to −2 mV. Fig. 11*A* shows examples of currents measured at three different pulse frequencies in the absence and presence of Bay K8644 (0.5 μM). Currents were always measured after steady state had been reached at a particular pulse rate. At low frequency (0.03 Hz), the drug increased peak inward current. This effect was minimal at intermediate frequencies (0.3 Hz). At higher pulse frequencies (3.0 Hz), the drug reduced inward current compared to control.

These results are summarized in Fig. 11*B*, which shows a plot of peak inward

current against test pulse frequency. In the absence of drug, there was a slight decrease in the current amplitude with frequency, but in the presence of Bay K8644, the change in drug activity from agonist to antagonist was very dramatic.

Nisoldipine and other dihydropyridine Ca channel antagonists also exhibit use-dependent blocking activity, but only at frequencies higher than 2 Hz (when measured from similar holding potentials). This is roughly 10 times the frequency at which inhibition develops in the presence of Bay K8644, which suggests

TABLE II

Bay K8644: Shift in Inactivation

Purkinje fibers Preparation	Concentration of Bay K8644	Pulse duration	Shift
		s	*mV*
340-1	200 nM	30.0	−5.0
359-1	200 nM	0.5	−12.0
359-1	200 nM	10.0	−9.0
382-1	10 μM	0.5	−10.0
383-3	50 nM	0.5	−5.0
383-3	10 μM	0.5	−10.0
384-1	50 nM	0.5	−8.0
385-2	50 nM	0.5	−5.0
386-2	200 nM	0.5	−12.0
386-2	200 nM	10.0	−11.7
386-2	10 μM	0.5	−13.7
386-2	10 μM	10.0	−11.2

Data summary Concentration	Number of preparations	Mean shift	SEM
50 nM	3	−6	1.2
200 nM	3	−9.2	2.6
10 μM	3	−10.8	1.0

Single cells Cell	Concentration of Bay K8644	Pulse duration	Shift
		ms	*mV*
0685G9, A	500 nM	500	−10.4
0785A6, 7	500 nM	500	−11.1
0785BE, G	500 nM	500	−15.5
0785BK, M	500 nM	500	−9.8
		Mean ± SEM	−11.7±1.5

possible differences in underlying mechanisms. We thus carried out experiments to further compare the blocking actions of Bay K8644 and the previously characterized Ca antagonists.

Relative Block by Bay K8644 Depends on Pulse Voltage

Fig. 12 presents the results of a series of experiments designed to determine whether development of block by Bay K8644 depends on pulse voltage during repetitive depolarization. The protocol, shown schematically in the inset of the

figure, resembles that used for similar tests of nisoldipine (Sanguinetti and Kass, 1984*a*, Fig. 7). The holding potential was fixed at −70 mV for 2 min and then changed to −55 mV. A train of repetitive pulses was applied to different conditioning voltages from the −55-mV holding potential. Membrane current was measured during a step to +2 mV after applying 14 conditioning pulses, and was compared with current measured during a single pulse to +2 mV that was not preceded by conditioning pulses. The magnitude of the peak inward currents measured using these two protocols in the absence and presence of Bay K8644 was used to plot the bar graphs shown in the figure.

In the control experiments (open bars), the trains of voltage pulses had little influence on measured currents, regardless of conditioning potential. In the presence of drug (striped bars), the peak current measured with the single-pulse

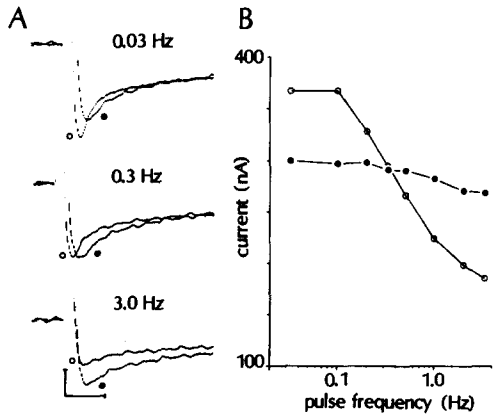


FIGURE 11. Effect of pulse frequency on activity of Bay K8644. Membrane current was measured in response to 50-ms pulses to −2 mV from a −66-mV holding potential. Pulses were applied at several frequencies. (*A*) Currents in the absence (●) and presence (○) of 500 nM Bay K8644, measured in response to pulses applied at 0.03, 0.3, and 3 Hz. (*B*) Peak current is plotted against pulse frequency. Lines are hand-drawn. 5.4 mM Sr. Fiber 352-2.

protocol was increased more than twofold over control and the conditioning voltages had marked effects on membrane current. The current measured after repetitive pulsing was always diminished relative to the single-pulse current, and this effect became more pronounced as the conditioning voltage was made more positive. In this experiment, Bay K8644 did not reduce absolute current compared with predrug values. However, in the presence of Bay K8644, repetitive pulsing did decrease peak current compared with the single-pulse measurement, and this effect can thus be considered "relative block."

Thus, in this experiment, a change in holding potential from −70 to −55 mV was not sufficient to reverse the agonist actions of Bay K8644. Instead, relative block developed only after repetitive depolarization and depended on the voltage of the conditioning pulses. Similar results were obtained in two additional experiments.

DISCUSSION

Contrasts Between the Actions of Bay K8644 and Ca Antagonists

The principal new results we present in this article reveal multiple actions of the Ca channel current agonist Bay K8644. Furthermore, we carried out experiments

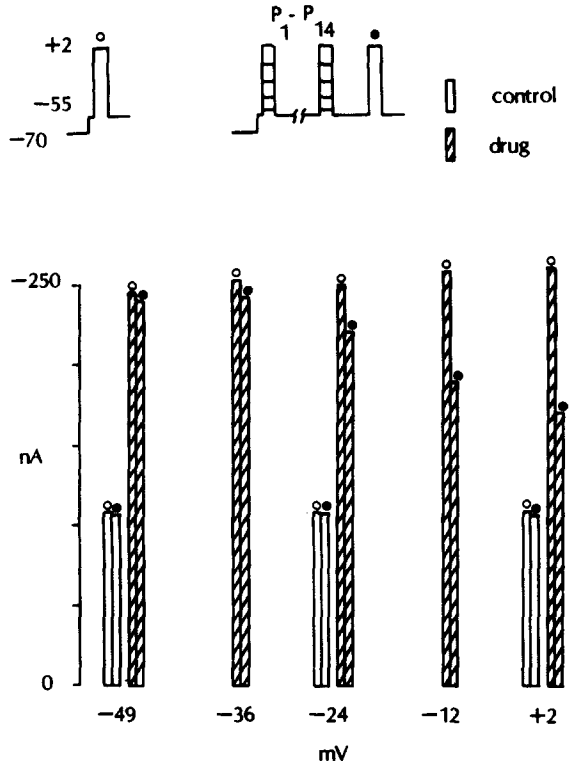


FIGURE 12. Influence of pulse voltage on actions of Bay K8644 during repetitive activity. Voltage protocols are shown in the inset. The holding potential was changed from −70 to −55 mV. Current was then measured in response to a single test pulse (○) and in response to a test pulse after applying a train of 14 pulses (P_1–P_{14}), 10 ms in duration, at 1.0 Hz to conditioning potentials (●). The absolute values of currents measured using these protocols are shown as bars for control (open) and drug (striped) conditions and are plotted against conditioning pulse voltage. 5.4 mM Sr. Fiber 353-1.

in multicellular (calf Purkinje fiber) and enzymatically dispersed single cell (rat ventricle) preparations and obtained similar results in each case. This drug speeds the rate of inactivation of Ca channel currents measured in both Purkinje fibers and single cells during depolarizing voltage pulses when Ca^{2+} or Sr^{2+} is the permeant ion. Further, currents appear to inactivate faster even when current amplitude is reduced in the presence of the drug (see Figs. 8 and 10). Thus, this action of the drug is not caused by enhancement of Ca-dependent inactivation by currents of larger amplitude. Bay K8644 also shifts the Ca channel activation

curve in the hyperpolarizing direction. Frequency-dependent inhibition of Ca channel currents develops at pulse frequencies near 0.2 Hz in the presence of Bay K8644, and the development of relative block depends on the pulse voltage during repetitive activity. Finally, Bay K8644 causes hyperpolarizing shifts in the inactivation vs. voltage relationship for Ca channel current after the application of 500-ms conditioning pulses.

This is in contrast to results obtained with nisoldipine (and other dihydropyridine Ca antagonists that are predominantly in the neutral form at physiological pH). The blocking activity of nisoldipine is very sensitive to changes in holding potential, but is not well correlated with pulse voltage during repetitive depolarization. The presence of nisoldipine causes a large hyperpolarizing shift of the steady state inactivation relationship in the absence of repetitive activity, an action that is dependent on both the concentration and the prepulse duration. If conditioning pulses are 500 ms in duration, nisoldipine does not shift inactivation (Sanguinetti and Kass, 1984*a*; see also Uehara and Hume, 1985). Finally, the dihydropyridine Ca channel antagonists do not shift the peak of the Ca channel current-voltage relationship and thus do not affect the Ca channel activation curve as does Bay K8644 (Lee and Tsien, 1983).

Taken together, the differences in the actions of these drugs suggest to us that the blocking activity of Bay K8644 results from a mechanism distinct from that responsible for Ca channel current block by nisoldipine and other classical dihydropyridine antagonists.

Does Bay K8644 Block Channels in the Open State?

The correlation between the development of relative block by Bay K8644 and pulse voltage, as well as the enhanced rate of inactivation in the presence of the drug, raises the possibility that Bay K8644 blocks channels in the open state. We feel that is an unlikely mechanism for at least two reasons. First, data from single channel currents are not consistent with open channel block. Channel open times are prolonged by the drug (Ochi et al., 1984; Hess et al., 1984). Second, open channel block, by itself, cannot explain the hyperpolarizing shift of the voltage dependence of channel activation and inactivation.

Block by Bay K8644: a Simple Model

We propose an alternative mechanism to explain the actions of Bay K8644 that incorporates published results obtained from single channel experiments. At the single channel level, the most consistent effect of Bay K8644 has been to cause an increase in the mean open time of Ca channels (Hess et al., 1984; Ochi et al., 1984; Kokubun and Reuter, 1984). Although Brown et al. (1984) have suggested that this change in channel kinetics occurs only at high (micromolar) drug concentrations, Hess et al. noted long openings in the presence of 100 nM Bay K8644, and Ochi et al. reported long openings with as little as 20 nM Bay K8644. We used a simple kinetic model for the Ca channel to examine how changes in mean open time at the single channel level might translate into effects observable at the macroscopic level. Specifically, we sought to reconstruct changes in macroscopic currents that we had observed in our experiments.

The model used for channel kinetics is diagrammed below. For simplicity, the channel was assumed to have three states: resting, open (conducting), and inactivated. The only allowable transitions were between resting and open or open and inactivated states (see diagram). No transitions between resting and inactivated states were assumed. The rate constants were chosen to be consistent with experimentally determined activation and inactivation curves, as well as (within the limits of the model) inactivation kinetics (see Kass and Sanguinetti, 1984). The voltage dependence was assigned only to the rate constants between the resting and open states, similar to models describing nerve Na channel kinetics (Armstrong and Bezanilla, 1977).

$$\mathrm{R} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \mathrm{O} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \mathrm{I} \qquad (1)$$

Single channel studies have shown that Bay K8644 increases mean open time at voltages where the averaged drug-containing records show no time-dependent inactivation. For a sequential model such as the one we are using, mean open time (t_o) is given by $t_o = (k_{21} + k_{23})^{-1}$. Since little or no inactivation occurs, k_{21} is the dominant rate constant affecting mean open time. Thus, k_{21} must be decreased to cause an increase in t_o. Such an effect has been previously suggested by Ochi et al. (1984). Therefore, we decreased the rate constant k_{21}, and chose

FIGURE 13. (*opposite*). Reconstruction of the effects of Bay K8644 with a kinetic model. A three-state model (Eq. 1) was used to describe Ca channel kinetics. The equations describing the occupancy of the states, along with the general solution for the functional occupancy of the open state, are the same as those given by Bennett et al. (1985). Rate constants were assigned within the constraints noted in the text. O, control conditions; ●, drug. (*A*) Simulation of membrane current in response to a voltage step from −60 to −8 mV in the absence and presence of Bay K8644. (*B*) Simulation of currents in response to test pulses to −8 mV preceded by prepulses to −60, −40, −20, and −8 mV (the simulated current becomes progressively less inward as prepulse voltage is made more positive). (*C*) Inactivation curves obtained from simulated peak currents in control and drug. Rate constants (in ms^{-1}) in control were:

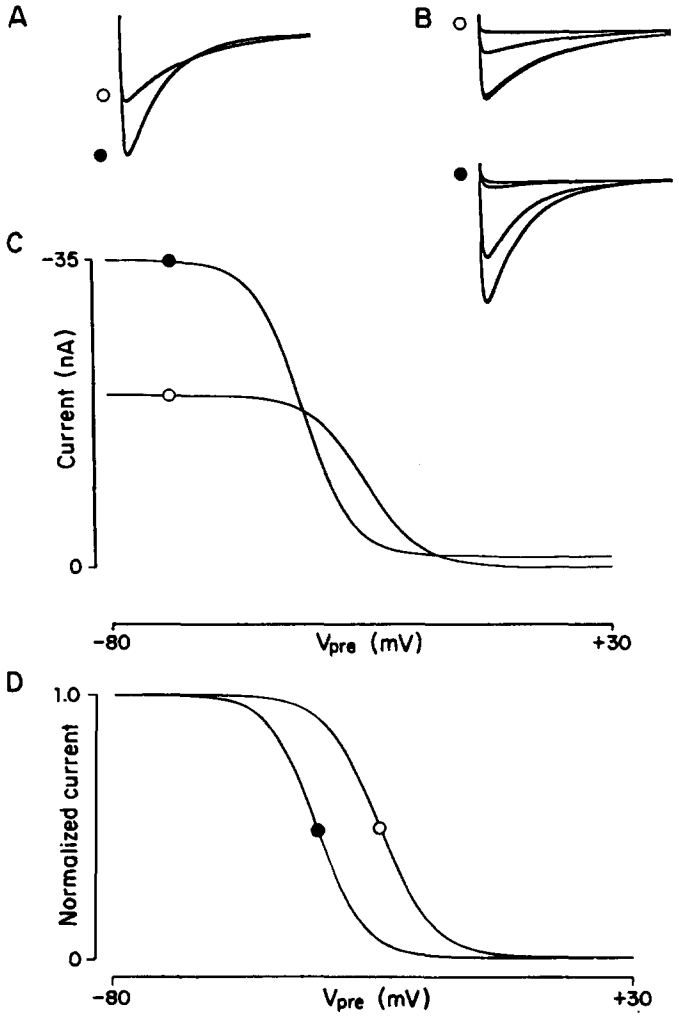
V_m	k_{12}	k_{21}	k_{23}	k_{32}
mV				
−40	0.0038	0.75	0.05	0.005
−20	0.10	0.67	0.05	0.005
−8	0.62	0.62	0.05	0.005
0	2.06	0.58	0.05	0.005
+20	49.5	0.5	0.05	0.005

The addition of Bay K8644 was assumed to decrease k_{21} by a factor of 10 at all voltages, without any other effects. For the Bay K8644 simulation, an additional calculation was done for a voltage of −60 mV. The rate constants at that voltage were assumed to be as follows (in ms^{-1}): $k_{12} = 0.0002$, $k_{21} = 0.09$, $k_{23} = 0.05$, and $k_{32} = 0.005$. (*D*) Curves in *C* normalized to maximum currents in the absence and presence of drug.

to decrease it by a factor of 10 to simulate the effects of Bay K8644 on single channel currents (see, for example, Hess et al., 1984). It should be emphasized that this is the only change that we have assumed for drug-containing conditions.

Fig. 13 shows that several effects are seen in macroscopic current as a consequence of making this change in the computation. Each of these effects, generated theoretically here, is consistent with our experimental observations. First, as indicated in panel *A*, reducing k_{21} by a factor of 10 increases the peak current magnitude, as well as the rate of inactivation of current measured during a test pulse applied after a negative conditioning potential (or from a negative holding potential). The currents simulated in this panel are in response to a test pulse to −8 mV applied from a −60-mV holding potential before and after decreasing k_{21}.

Second, the decrease in k_{21} in our model changes the relationship between Ca



channel availability and membrane potential. Panel *B* shows two sets of records simulating measurements of currents in the absence (open circles) and presence (solid circles) of drug. Currents were simulated during a pulse to −8 mV after prepulses to −60, −40, −20, and −8 mV. The simulation shows that for negative prepulses, the peak inward current in the presence of drug is larger than control; however, after positive prepulses, currents in the presence of Bay K8644 are smaller than control.

This dual action of Bay K8644 causes a "crossover" of the unnormalized inactivation curve in the absence and presence of drug as seen in *C* (see Fig. 7 for experimental curves). The normalized inactivation curve is shifted in the hyperpolarizing direction in the presence of Bay K8644, as is shown for the computational results in *D*, and for the experimental data in Figs. 10 and 11. The "crossover" effect on currents explains why Bay K8644 can act as an agonist under some experimental conditions and as an antagonist under other conditions, as has been previously reported (Sanguinetti and Kass, 1984*b*).

As a result of these calculations, we feel that the Ca channel current inhibition seen with Bay K8644 does not necessarily imply that this drug shares a common mechanism of block with other dihydropyridines that are primarily antagonists. Our model suggests that the antagonism seen in the presence of Bay K8644 at more positive potentials is a direct consequence of the same kinetic change that produces agonist effects at more negative membrane potentials and is not due to an additional effect of the drug.

Shortcomings of the Model

Two aspects of the actions of Bay K8644 are not explained by our model. First, we found that relative block by Bay K8644 can develop in a use-dependent manner (Fig. 11). Although the drug-induced speeding of inactivation that is predicted by the model contributes to the "accumulation" of inactivation with time in these experiments, the rate of recovery from inactivation is very important to the development of use-dependent block. We did not incorporate recovery from inactivation into our modeling, nor did we test for use-dependent effects. Thus, we cannot claim to explain this action of Bay K8644 by our kinetic model.

In our experiments, we did not quantitatively examine the concentration dependence of the various actions of Bay K8644, but the data summarized in the tables suggest that the shifts in the voltage dependence of activation and inactivation appear to be concentration dependent. There is such a trend for the peaks of the current-voltage relationships shown in Fig. 1, although, as pointed out in the text, the resolution of small changes in the locations of the peaks in these curves is difficult. Current amplitude also varied with drug concentration in the experiment of Fig. 1 and, in addition, it was possible that at the higher drug concentrations, current amplitude continued to increase after the shift in the peak of the current-voltage relationship had saturated.

We found that we could simulate a concentration-dependent change in both the voltage dependence and the amplitude of currents by varying the Bay K8644–dependent rate constant k_{21} in our model. We decreased this rate constant by factors of 2, 5, 10, 50, and 100 and obtained graded changes in the voltage

dependence of activation and inactivation, as well as in the magnitude of peak current. As the rate constant was made very small, both the shift and change in amplitude saturated.

Thus, although this simple model accounts qualitatively for most of our experimental observations on macroscopic currents, it cannot explain a possible enhancement of the macroscopic current amplitude in the absence of a shift in the voltage dependence of activation or inactivation. Our computations show that either a change in a more complicated scheme is needed to account for such a separation, or that Bay K8644 causes effects in addition to the prolongation of mean open times of the channel. One way in which the drug could cause such an effect would be to reduce the probability that the channel will fail to open during depolarization, in addition to affecting the mean open time of channels that do open. A direct test of this possibility can be carried out with experiments on single channel currents.

Predictions of the Model

One future test of this model will be to examine the effects of Bay K8644 on Ca channel mean open times over a wider potential range than has previously been studied. Published data that demonstrate an increase in mean open time have been obtained at voltages and under experimental conditions where currents show little or no inactivation. At voltages where control or drug-containing currents do inactivate, our model predicts that channel open times should still be prolonged, since the only change caused by the drug is a decrease in one of the rate constants for leaving the open state. This change in open time should still occur despite the fact that the probability of the channel being in the open state decreases as inactivation occurs.

Relationship to Previous Work: Multiple Types of Ca Channels and Different Modes of Gating

Several recent studies have demonstrated that at least two Ca channel types may exist in isolated dorsal root ganglion cells (Nowycky et al., 1985), smooth muscle cells (Fox et al., 1985), GH_4C_1 pituitary cells (Cohen and McCarthy, 1985), and cardiac cells (Bean, 1985; Nilius et al., 1985). In all cases, a rapidly inactivating channel that is activated at relatively negative test potentials (T channel) is unaffected by dihydropyridine agonists or antagonists. The activity of another Ca channel type, which inactivates more slowly and is activated at more positive test potentials (L channel), is blocked by dihydropyridine antagonists and enhanced by Bay K8644.

It is unlikely that the voltage-dependent effects of Bay K8644 in the present experiments can be explained simply by differential effects on two Ca channel types. The most obvious effects of Bay K8644 in our preparations are: (*a*) preferential enhancement of current during test pulses to potentials less negative than 0 mV; (*b*) an increased rate of apparent Ca channel current inactivation; and (*c*) holding potential–dependent modulation of current, which could be interpreted as preferential activation of T channels in the presence of the drug. This interpretation would be contrary to all of the studies cited above, where it

is quite clear that T-type channels are unaffected by Bay K8644. Therefore, assuming that multiple Ca channels do exist in the Purkinje fiber, we suggest that the effects observed in the present study represent changes in the gating of L-type, and not T-type, channels. This suggestion is also consistent with the observations that T-type channels contribute very little to the total Ca channel current in ventricular cells (Bean, 1985; Nilius et al., 1985).

The racemic mixture of a new dihydropyridine derivative, 202-791, has been shown to either enhance or inhibit contraction of isolated rabbit aortic rings after depolarizations caused by low or high levels of K, respectively (Hof et al., 1985). The agonist activity is associated only with the S-enantiomer and antagonist activity only with the R-enantiomer in this vascular smooth muscle preparation. We used racemic Bay K8644 in all of our experiments. It is not likely that the mixed agonist effects of Bay K8644 are simply due to opposing actions of two stereoisomers, because preliminary experiments with the S-enantiomer of 202-791 shows that this reportedly pure agonist also exhibits voltage-dependent mixed agonist effects similar to those observed with Bay K8644 (Sanguinetti, M. C., unpublished observation), but more complete experiments are needed to verify these results.

Previous studies have discussed the actions of Bay K8644 and other dihydropyridines in terms of stabilization of different modes of channel gating (Hess et al., 1984; Fox et al., 1985), which is a conceptually useful way to think about the modulation of channel activity that occurs on a relatively slow time scale. We have taken a different approach and tried to explain the effects of Bay K8644 in terms of a simple kinetic model that specifically addresses the question of what changes may have occurred at the level of channel gating. One theory is not necessarily exclusive of the other. In fact, the kinetic change we describe may be at the heart of the transition from short to long channel openings noted in the above studies (i.e., mode 1 to mode 2). One substantial difference between our interpretation of the mechanism of action of Bay K8644 and that in earlier studies is that the drug itself (or an endogenous analogue in the absence of drug) causes the rate constant change in our model rather than stabilizes an already existing change (see Fox et al., 1985). The fact that changing a single rate constant in the kinetic model can account for the complex macroscopic effects of Bay K8644 makes our idea an attractive one in explaining the mechanism of action of this drug.

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