Calcium Channel Antagonist Properties of Bay K 8644 in Single Guinea Pig Ventricular Cells

Robert W. Hadley and Joseph R. Hume

The effects of Bay K 8644 on the high-threshold calcium channel was investigated by means of the whole-cell patch-clamp technique in single guinea pig ventricular myocytes. The goal of the experiments was to characterize the inhibitory effects of Bay K 8644 on the calcium channel, and identify the factors that influence the inhibition. Bay K 8644 was found to have strong calcium channel antagonist properties, which were both dose- and voltage-dependent. Channel block by Bay K 8644 had both a tonic and a use-dependent component. The stimulatory effect of the drug was found to have little obvious dependence on the holding potential. The accumulation of use-dependent block during trains of pulses was facilitated by faster rates of stimulation, longer pulse durations, and more positive holding potentials. Application of the drug induced the appearance of a second, slow component of calcium channel recovery. Both the time-constant and relative proportion of the slow component of recovery were found to be voltage-dependent. Bay K 8644 was also found to cause a hyperpolarizing shift of the inactivation curve for the calcium current, suggesting that it has strong interactions with the inactivated state of the calcium channel. Thus, Bay K 8644 has, along with its stimulatory effects, inhibitory effects that strongly resemble those of typical calcium channel antagonists. (Circulation Research 1988;62:97-104)

Bay K 8644 is a dihydropyridine that can, among other effects, constrict blood vessels and increase the force of contraction of the heart.1 The drug is thought to increase the entry of calcium into cells by increasing the probability of calcium channels being open, either by prolonging the duration of individual openings2-3 or by increasing the number of openings.8

It was noted in early experiments with Bay K 8644 that the dose-response curve was biphasic. The increase in muscle contractility peaked in the micromolar range and then declined at higher concentrations.1 This observation has led to models of the calcium channel with multiple dihydropyridine binding sites with different binding properties and functions.6-7 Calcium channel inhibition by Bay K 8644 has also been detected on the electrophysiological level as block of the calcium current (I Ca).1 Bay K 8644 has been shown to be strongly voltage-dependent in a manner similar to that of other dihydropyridines.6-9 However, it has also been reported that the effects of Bay K 8644 on I Ca are chiefly stimulatory at depolarized holding potentials.5,10

This paper describes an investigation that has been made to quantitatively assess the effects of Bay K 8644 on I Ca in single guinea pig myocytes. The relative importance of calcium channel agonist or antagonist activity is shown to depend on drug concentration and membrane potential, and the antagonist properties of Bay K 8644 are shown to induce important changes in channel properties.

Materials and Methods

Cell Preparation

The single guinea pig ventricular cells were isolated by a collagenase dispersion technique as has been previously described.11

Recording Techniques

The myocytes were voltage-clamped using the whole-cell patch-clamp technique.12 Pipettes have resistances of 1-3 MΩ after they were fire polished and then filled with the standard internal solution. Voltage-clamp commands were supplied from a Dagan 8900 patch-clamp amplifier. Series resistance compensation was used in all of the experiments.

Solutions

After the myocytes were placed in the experimental chamber, they were perfused with a solution containing (in mM): NaCl 144, KCl 5.4, CaCl 2 2.5, MgCl 2 0.5, glucose 5.5, and N-2-hydroxyethylpiperazine-N2-2-ethanesulfonic acid (HEPES) 5. The perfusate was gassed with 100% O 2 and was kept at room temperature (20-22°C). The internal (pipette) solution consisted of (in mM) Cs aspartate 110, CsCl 20, tetraethylammonium Cl 20, ethyleneglycol-b-aminobutyric acid (EGTA) 5, HEPES 5, and Mg ATP 1. The external solution was changed to a sodium-free solution after equilibration of the pipette solution with the cytoplasm had taken place. The sodium-free solution contained (in mM) Tris Cl 129, CaCl 2 20, KCl 5.4, CaCl 2 2.5, MgCl 2 0.5, glucose 5.5, and tetrodotoxin 0.01. Potassium currents were reduced effectively
by the presence of cesium ions on both sides of the membrane as well as tetraethylammonium ions intracellularly. $I_C$ was always measured before and after block by CdCl$_2$ (500 μm). Bay K 8644 [methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl-ethylammonium)-pyridine-5-carboxylate] was dissolved in polyethylene glycol to make stock solutions, and aliquots of this were added to the perfusate as required. Equivalent aliquots of the diluent alone had no observable effect on $I_C$. To obtain steady-state effects, the myocytes were exposed to Bay K 8644 for 5–10 minutes before the experiments.

It should be noted that calcium channel rundown was not accounted for in the calculations of absolute drug-induced changes in $I_C$ magnitude. We, along with other investigators, have found that the inclusion of Mg-ATP in the pipette solution slows this process to acceptable levels. Great care was taken in minimizing this error by selecting cells that had a stable control $I_C$ over several minutes of observation. Naturally, rundown should have little effect on the recovery protocols, as each test $I_C$ is compared with the $I_C$ during the prepulse.

**Data Analysis**

Data was recorded on a tape recorder (model 4DS, Racal Recorders, Southampton, United Kingdom) and then was digitized at an interval of 0.25–2 msec on a PDP 11-23 computer, where it was analyzed. Voltage and current traces were displayed with a digital plotter. The time course of calcium channel recovery was described with one or two exponentials by a least-squares fit to the equation $y = A_0 - A_1 \exp(-t/t_1) - A_2 \exp(-t/t_2)$. In this equation, $A_0$ is a constant, $t$ is time, $t_1$ and $t_2$ are the time constants of recovery, and $A_1$ and $A_2$ are the preexponential coefficients for their respective time constants.

**Results**

Figure 1 shows examples of tests that have been made of the quality of $I_C$ isolation. Panel A shows current traces before and after 500 μM CdCl$_2$ was added to the perfusate. The cell was held at −80 mV and then stepped to 0 mV. A large $I_C$ was evoked under control conditions but was completely blocked by cadmium, leaving only time-independent current. It should be noted that the zero-current level of cells under these conditions is only 20–30 pA outward to the holding current. This result, along with those of a previous study, suggests that $I_C$ has been satisfactorily isolated. However, the use of fairly negative holding potentials raises the possibility that low-threshold calcium channels may contribute to $I_C$. This was tested by blocking $I_C$ with nifedipine, as low-threshold calcium channels are less insensitive to dihydropyridines. Nifedipine blocked almost all of the $I_C$, as shown in Panel B, except for a small rapidly inactivating component of approximately 20 pA. This may be current through low-threshold calcium channels, but it appears to make a negligible contribution to ventricular $I_C$ under these conditions, as reported by other investigators. It also should be noted that application of 2 mM 4-aminopyridine did not affect $I_C$ (Panel C), indicating that a transient outward current ($I_O$) is not present under these experimental conditions. Thus, the membrane current shown in these records is predominantly through high-threshold calcium channels.

The initial studies with Bay K 8644 were aimed at defining the dose dependence of its effects. Figure 2 shows representative records of $I_C$ evoked by 500-msec voltage-clamp steps to 0 mV from two holding poten-
perpolarizing pulses produced only small changes in dependent and calcium-dependent inactivation. Hy-

Table 1. Dose Dependence of Bay K 8644

<table>
<thead>
<tr>
<th>Concentration of Bay K 8644</th>
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<tbody>
<tr>
<td></td>
<td>25 nM</td>
</tr>
<tr>
<td>−40 mV</td>
<td>−12.0 ± 9.8</td>
</tr>
<tr>
<td>−80 mV</td>
<td>−58.6 ± 6.6</td>
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</table>

*Mean percent change in peak lca is shown with the SEM, n = 6.

The acceleration of calcium channel inactivation by Bay K 8644 has been noted by other investigators. However, it is possible that the acceleration of decay in this study could be a secondary effect due to the enhanced calcium entry. This possibility was tested by adding a partially blocking concentration of cadmium (25 μM). The lca magnitude was diminished greatly, but lca inactivation, as measured by the half-time for decay, was still quite fast. In a total of five cells, the average half-times for decay were 17.2 ± 2.9 msec for control, 6.2 ± 1.6 msec for Bay K 8644, and 6.0 ± 1.1 msec for Bay K 8644 and cadmium.

To more closely examine the inhibition of lca by Bay K 8644, it was essential to examine its effects over a wide range of potentials. In these experiments, 250 nM was chosen as the usual concentration of Bay K 8644 because it produced a moderate inhibition. Figure 3A shows the change in peak lca induced by Bay K 8644 at holding potentials from −90 to −30 mV. The cell was held from 10 to 20 seconds at each potential before a test pulse was given. On average, Bay K 8644 more than doubled the current size at negative potentials, but this effect was somewhat smaller at −50 mV and abruptly turned into a strong inhibitory effect at −40 mV, which is usually the threshold for lca activation and inactivation in these cells.

A second method of examining the voltage dependence of the drug effect was to look at the inactivation curve of lca. Figure 3B shows the inactivation curve obtained under control conditions and after application of 250 nM Bay K 8644. The curve was obtained with a two-pulse protocol: a 500-msec test pulse to 0 mV was separated by 10 msec from a 500-msec prepulse to a variety of potentials. The holding potential was −50 mV. The control inactivation curve had a partial U-shape due to the respective roles of voltage-dependent and calcium-dependent inactivation. Hyperpolarizing pulses produced only small changes in the test current, which is an indication of the small role of the low-threshold calcium channel. Bay K 8644 induced several changes in the inactivation curve. The curve was shifted in a hyperpolarizing direction, and its slope was more gradual. The apparent Vm for the control curve was −19 mV, and Bay K 8644 shifted Vm to −36 mV. Bay K 8644 also seemed to partly suppress the upturn of the inactivation curve at positive potentials. Such drug-induced changes in the inactivation curve have been noted with other dihydropyri-

![Figure 2. Dose dependence of stimulatory and inhibitory effects of Bay K 8644 on lca. Top of each panel shows voltage-clamp protocol, where a 500-msec step to 0 mV is given from a holding potential of −80 mV (left panel) or −40 mV (right panel). Bottom of each panel shows current records under control conditions and at different concentrations of Bay K 8644. Cell was stimulated at 0.1 Hz.](http://circres.ahajournals.org/)

![Figure 3A.](http://circres.ahajournals.org/)

![Figure 3B.](http://circres.ahajournals.org/)
FIGURE 3. Voltage dependence of effect of Bay K 8644 on I_Ca. Panel A, average percent change (with SEM) in peak I_Ca magnitude induced by 250 nM Bay K 8644 at different holding potentials. The holding potential was allowed to remain at the new value for 10–20 seconds before I_Ca was evoked by a 200-msec step to 0 mV. Panel B, inactivation curves before (*) and after (□) applying Bay K 8644. See text for voltage protocol. x axis, potential of the inactivating prepulse; y axis, relative magnitude of I_Ca during the subsequent test pulse. Curves through the data points were drawn by eye.

bottom half of Figure 4A shows the time course of the change in Bay K 8644's effect. It can be described by a single exponential with a time constant of 3,132 msec. An analogous experiment is shown in Figure 4B, where the cell was held at -50 mV, and the time course of the increase in current size upon stepping to -80 mV was determined. In the example shown, the time course could be described by an exponential of 356 msec. For a total of four cells, the average time constants were 3,038 ± 443 msec (SEM) at -50 mV and 533 ± 108 msec at -80 mV.

Because it has been shown that depolarization of the cell membrane accentuates Bay K 8644's inhibitory effects on I_Ca, it would make sense that the more frequently the cell is depolarized, the more prominently the inhibitory effects would be seen. An example of this is shown experimentally in Figure 5, as the drug-induced change in I_Ca is plotted against the frequency of stimulation for two different holding potentials. A tenfold increase in the rate of stimulation somewhat diminished the stimulatory effect of Bay K 8644 at a holding potential of -80 mV. A more prominent change is seen at -50 mV, where an increase in the rate of stimulation altered Bay K 8644's effect from being net stimulatory to net inhibitory. It should be noted that the effects of changing the stimulation rate were somewhat variable from cell to cell, in that increasing the frequency always enhanced the inhibitory effects, but the frequency at which the net effect changed from being stimulatory to inhibitory was different among cells. Changing the stimulation rate in the absence of drug caused a decrease in I_Ca magnitude of 11% (0.33 Hz) and 53% (1.0 Hz) at a holding potential of -50 mV and 0% (0.33 Hz) and 9% (1.0 Hz) at a holding potential of -80 mV, relative to I_Ca at 0.1 Hz.

As the inhibitory effect of Bay K 8644 appeared to be strongly use-dependent, it would be useful to study the onset of the use-dependent block and to determine what factors influence it. It has already been established that calcium channel block by Bay K 8644 is accentuated by more positive test pulses. Figure 6A shows

FIGURE 4. Kinetics of tonic block by Bay K 8644. Cell was exposed to 250 nM Bay K 8644. Upper half of Panel A shows the voltage protocol for determining time course of onset of tonic block at -50 mV. Bottom half of Panel A plots magnitude of test I_Ca against duration of prepulse to -50 mV. Time course of onset of tonic block was fit with a single exponential of 3,132 msec. Upper half of Panel B shows an analogous voltage-clamp procedure for determining time course of removal of tonic block. Time course of recovery is plotted in bottom half of Panel B and can be fit with a single exponential of 356 msec.
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FIGURE 5. Increased frequency of stimulation increases inhibitory effects of Bay K 8644. Figure shows example of the effect altering stimulation frequency (x axis) has on the net change in $I_{Ca}$ magnitude (y axis) induced by 250 nM Bay K 8644. Data points are steady-state effects seen with 200-msec pulses to 0 mV from two different holding potentials, −50 mV (○) and −80 mV (●).

examples of the response of $I_{Ca}$ after a rest period, to a train of depolarizations to 0 mV (see figure legend for details). Under control conditions at −80 mV, $I_{Ca}$ responded to 20-msec depolarizations at 0.33 or 1 Hz with a small positive staircase effect. If Bay K 8644 was present, $I_{Ca}$ decreased in response to repetitive stimulation. (Both control and Bay K 8644 currents are scaled to show the onset of changes in $I_{Ca}$ with time.) Faster stimulation produced more block. Figure 6B shows that increasing the duration of the test pulse to 200 msec caused the use-dependent block to accumulate to a greater extent. Figure 6C shows that a more depolarized holding potential, −50 mV, had the same effect. It is also apparent that it takes more pulses for the block to approach steady state when the test pulse was widened or the cell held more positive.

Since Bay K 8644 is known to accelerate $I_{Ca}$ inactivation, it was of interest to examine its effects on recovery from inactivation. Figure 7A shows the time course of recovery at −50 mV under control conditions (marked by triangles), obtained with a protocol that consisted of two 500-msec pulses to 0 mV that were separated by a variable interval. The magnitude of the test $I_{Ca}$ was then compared with the magnitude of $I_{Ca}$ during the first pulse. The recovery at −50 mV was characterized by a single time constant of 422 msec. The circles in the same panel show the recovery after Bay K 8644 was applied. The recovery was best characterized as a two-exponential process, with a fast time constant of 400 msec and a much slower one of 3,924 msec. Figure 7B shows the control and drug-influenced $I_{Ca}$ recovery at a holding potential of −80 mV. The control recovery had a time constant of 93 msec. Bay K 8644 once again slowed $I_{Ca}$ recovery, as its time course could be described by time constants of 130 msec and 697 msec.

Table 2 summarizes the recovery data from six cells. It can be seen that Bay K 8644 induced a second, slower exponential of recovery that appears to have a voltage dependence that was similar to that of the control recovery. A comparison of $I_{Ca}$ recovery at the two potentials investigated shows that at −50 mV, the drug-induced time constant was not only slower but also accounted for more of the recovery process.

Although it has been demonstrated in guinea pig ventricular myocytes that a moderate amount of calcium entering the cell does not slow recovery, there are nevertheless some reports of this phenomenon in other preparations. Figure 8 shows a test that was made to ensure that the slowed recovery process was not secondary to the enhancement of calcium entry by...
Bay K 8644. Figure 8A shows the slowed recovery at -80 mV observed in a myocyte after application of Bay K 8644. The time course was described as a biexponential process with time constants of 117 msec and 1,012 msec. Figure 8B shows the same cell when 25 μM Cd was added to the Bay K 8644-containing solution. The time constants of recovery were quite similar, with values of 102 msec and 1,280 msec. The magnitude of the slow component seemed to be enhanced, but it is at least certain that reducing calcium entry did not eliminate the slowed \( I_{Ca} \) recovery.

**Discussion**

The results presented here have shown convincingly that inhibitory effects are a prominent feature of the net effect of Bay K 8644 on \( I_{Ca} \) in the heart. These studies have provided new, quantitative data on the voltage and concentration dependence of Bay K 8644's stimulatory and inhibitory effects on the kinetics of channel block and unblock, on the factors that influence the accumulation of use-dependent block, and on drug-induced changes in calcium channel repriming.

An important question that needs to be addressed is the relative importance of the two enantiomers of Bay K 8644. It has been reported that the activity of the two enantiomers is differentiable, with (-)-Bay K 8644 having chiefly stimulatory effects and (+)-Bay K 8644 having principally inhibitory effects. Similar observations have also been seen with the stimulatory dihydropyridine 202-791. Racemic Bay K 8644 was used in all of the experiments shown in this paper, but it is necessary to evaluate the importance of the two enantiomers to the inhibitory effects described here. It would seem likely that (+)-Bay K 8644 would make a strong contribution to the inhibitory effects, and an examination of the dose-response data presented in

**Table 2. Effect of Bay K 8644 on \( I_{Ca} \) Recovery**

<table>
<thead>
<tr>
<th>Condition</th>
<th>( V_h ) (mV)</th>
<th>( n )</th>
<th>( t_1 ) (msec)</th>
<th>( t_2 ) (msec)</th>
<th>( A_1 )</th>
<th>( A_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-50 mV</td>
<td>6</td>
<td>337 ± 56</td>
<td>368 ± 94</td>
<td>0.31 ± 0.05*</td>
<td>0.54 ± 0.07*</td>
</tr>
<tr>
<td>Control</td>
<td>-80 mV</td>
<td>6</td>
<td>104 ± 9</td>
<td>368 ± 94</td>
<td>0.31 ± 0.05*</td>
<td>0.54 ± 0.07*</td>
</tr>
<tr>
<td>Bay K 8644</td>
<td>-50 mV</td>
<td>5</td>
<td>368 ± 94</td>
<td>3,077 ± 266</td>
<td>0.31 ± 0.05*</td>
<td>0.64 ± 0.07*</td>
</tr>
<tr>
<td>Bay K 8644</td>
<td>-80 mV</td>
<td>5</td>
<td>123 ± 10</td>
<td>866 ± 85</td>
<td>0.81 ± 0.01*</td>
<td>0.19 ± 0.03*</td>
</tr>
</tbody>
</table>

The concentration of Bay K 8644 that was used was 250 nM. \( t_1 \) and \( t_2 \) are the fast and slow time constants of recovery respectively. \( A_1 \) and \( A_2 \) are the relative amplitudes of the fast and slow phases of recovery, respectively.

*Significant difference between values in a column when compared with a Student's \( t \) test (\( p < 0.05 \)).
Table 1 suggests that this may be the case. The stimulatory effects seen at -80 mV seem to rise and peak at lesser concentrations than the inhibitory effects seen at a holding potential of -40 mV (Table 1). This is consistent with the possible importance of (+)-Bay K 8644 to the inhibitory effects, as this enantiomer has a somewhat lower affinity for the dihydropyridine receptor than (-)-Bay K 8644.26,27 However, the "calcium channel agonist" enantiomer of the stimulatory dihydropyridine 202-791, (+)-202-791, has been recently shown to have antagonist-like effects of its own.28,29 Also, clear evidence of concentration-dependent inhibitory effects of (-)-Bay K 8644 has been reported in smooth muscle from rat tail artery and guinea pig ileum.27 Therefore, it seems likely that (-)-Bay K 8644 also contributes to the inhibitory effects of the racemate.

It has been shown that part of the inhibitory effects of Bay K 8644 consists of a "tonic block" with moderate depolarizations. Kinetically, the process closely resembles the Bay K 8644-induced slow phase of recovery at both potentials. There was no significant difference between the time constants of recovery and the onset or offset of tonic block at either potential. This suggests that the processes are similar and that tonic block is due to active inhibition of channels rather than some unknown voltage dependence of the stimulatory effects, although the latter possibility cannot be completely excluded. This interpretation is supported by the lack of voltage dependence of the stimulatory effect over the potential range (-90 to -60 mV) where it predominates.

Bay K 8644 clearly induced a slow phase of recovery from inactivation, similar to classic calcium channel antagonists. A previous report indicated that Bay K 8644 hastened recovery of slow, calcium-dependent action potentials in rabbit papillary muscle.30 It is uncertain whether this difference may be due to a true difference between species or to the limitations of Vm as a measurement of ionic conductances.

It was shown that the Bay K 8644-induced slow recovery was not only strongly voltage-dependent, as has been seen with other calcium channel antagonists, such as D600,31 but that the voltage dependence appeared to be the same as that for normal recovery of the channels. This suggests that the processes are quite similar, with the channel having to proceed through a slowed recovery process before the drug molecule can either unbind or lose its inhibitory action. It was also found that the proportion of the slow phase of recovery was greatly accentuated by either holding the potential more positive or by the addition of a small concentration of cadmium to the perfusate. It was not surprising that a more positive holding potential increased the pool of channels that undergo slow recovery. However, the large increase in channels that undergo slow recovery seems out of proportion when compared to the modest amount of tonic block that was present at -50 mV. It almost seems as if the more positive holding potential promoted the additional channel block that occurred during the test pulse. The increase in the slow component of recovery that was caused by cadmium is more difficult to interpret. It is tempting, however, to speculate whether the effect of cadmium to promote Bay K 8644 block during the test pulse might be secondary to a decrease in ICa. It has been reported that millimolar concentrations of external calcium decrease the binding of an inhibitory dihydropyridine, (+)-PN 200-110, when the cells were polarized and a stimulatory dihydropyridine, (+)-202-791, was present.28 The conditions of the recovery experiments presented here were similar in that both stimulatory and inhibitory dihydropyridines, the enantiomers of Bay K 8644, were present. It might be possible that calcium must enter the channel to inhibit dihydropyridine binding, and this can be prevented by cadmium.

It seems easiest to interpret the drug-induced slow recovery phase as due to a stabilization of the inactivated state of the channel. This interaction with the inactivated state would seem to be able to account for most of Bay K 8644's inhibitory effects, although one recent study found little evidence for interactions with the inactivated state,28 and another proposed that the inhibitory effects of Bay K 8644 may be a consequence of its promoting the open state of the channel.3

In conclusion, Bay K 8644 has along with its stimulatory effect on ICa, strong inhibitory effects that may be related to an interaction with the inactivated state of the channel. An important question that remains is whether there are any fundamental differences between the inhibitory effects of racemic Bay K 8644 and (-)-Bay K 8644. Although it will be especially important to resolve this question for Bay K 8644 to continue to be a useful probe into the molecular structure and function of the calcium channel, other uses of racemic Bay K 8644 as an experimental tool should still prove productive, as experimental protocols can be carefully chosen so as to maximize the stimulatory effects and minimize the inhibitory effects of the drug.

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