# Fast activation of cardiac $Ca^{++}$ channel gating charge by the dihydropyridine agonist, BAY K 8644

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ABSTRACT Nonlinear charge movement (gating current) was studied by the whole-cell patch clamp method using cultured 17-d-old embryonic chick heart cells. $Na^+$ and $Ca^{++}$ currents were blocked by the addition of 10 $\mu$M TTX and 3 mM $CoCl_2$; $Cs^+$ replaced $K^+$ both intra- and extracellularly. Linear capacitive and leakage currents were subtracted by a P/5 procedure. The small size (15 $\mu$m in diameter) and the lack of an organized internal membrane system in these myocytes permits a rapid voltage clamp of the surface membrane. $Ca^{++}$ channel gating currents were activated positive to $-60$ mV; the rising phase was not distorted due to the system response time. The addition of BAY K 8644 ($10^{-6}$ M) caused a shortening of the time to peak of the $Ca^{++}$ gating current, and a negative shift in the isochronal $Q_{on}$ vs. $V_m$ curve. $Q_{max}$ was unchanged by BAY K 8644. The voltage-dependent shift produced by BAY K 8644 is similar to that produced by isoproterenol (Josephson, I. R., and N. Sperelakis. 1990. *Biophys. J.* 57:305a. [Abstr.]). The results suggest that the binding of BAY K 8466 to one or more of the $Ca^{++}$ channel subunits alters the kinetics and shifts the voltage dependence of gating. These changes in the gating currents can explain the parallel changes in the macroscopic $Ca^{++}$ currents.

## INTRODUCTION

The molecular mechanism of action of the dihydropyridine $Ca^{++}$ channel agonist, (−)BAY K 8644, has been pursued intensively (1, 2), yet it still remains unsolved. The electrophysiological effects of this agent have, however, been well-characterized on the macroscopic $I_{Ca}$, and on the single-channel calcium currents of both heart and smooth muscle cells. BAY K 8644 produces a concentration-dependent enhancement of the macroscopic $I_{Ca}$, shifts the steady-state activation and inactivation curves in the hyperpolarizing direction, increases the rate of activation of $I_{Ca}$, and slows its deactivation (3–7). At the single-channel level, the most obvious effect of BAY K 8644 is to dramatically prolong the $Ca^{++}$ channel open-time (5, 8–10). In addition, the latency to channel opening upon depolarization is shortened in the presence of BAY K 8644 (7, 8). To further explore the electrophysiological effects of BAY K 8644 on cardiac $Ca^{++}$ channels, and in particular to understand the nature of the voltage-dependent shift in channel gating, the effects of BAY K 8644 were tested on the $Ca^{++}$ channel charge movement (gating current) (16, 17), which initiates channel opening (14).

The cultured embryonic ventricular myocyte preparation was chosen for these experiments because it offered several advantages over the adult myocyte preparation with regards to recording and identifying the $Ca^{++}$ channel charge movement (12). The myocytes were small (ca. ~15 $\mu$m in diameter) and spherical, thereby permitting a fast-settling (<100 $\mu$s) voltage clamp. In addition, the relative scarcity of an organized internal membrane system reduced the possibility that a fraction of the charge movement signal arose from a source other than surface membrane channels.

## METHODS

### Cell culture preparation

Single ventricular cell cultures were prepared from 17-d-old embryonic chick hearts. In brief, two dozen fertilized White Leghorn chicks eggs were incubated for 17 d at 37.5°C and staged to confirm their degree of development. Hearts were removed by a sterile process and collected in a balanced salt solution (4°C). Tissue dissociation was accomplished by gentle rotation of the hearts in a $Mg^{++}$- and $Ca^{++}$-free Ringer solution containing 0.05% trypsin (Sigma Chemical Co., St. Louis, MO). The cell suspensions were harvested at 5-min intervals, pooled, and pelleted by centrifugation (85 *g*). The cells were washed three times, and resuspended in tissue culture medium (M199, Gibco Laboratories, Gibco Div, St. Lawrence, MA) containing 10% fetal bovine serum, and plated into 35 mm plastic petri dishes (Falcon Labware, Becton, Dickinson and Co., Winston Salem, NC) at a concentration of $10^5$–$10^6$ cells/ml. The myocyte cultures were maintained at 37°C and pH 7.4 in a moist-air $CO_2$ incubator (for 24–72 h) until used for experimentation.

### Recording

Single ventricular myocytes were voltage-clamped using the whole-cell configuration of the patch-clamp technique. Electrodes were fabricated from thin wall borosilicate glass (model TW-150, World Precision Instruments, Inc., New Haven, CT) and filled with the following solution (in millimolar): $Cs^+$, 140; glutamate, 140; Mg-ATP, 2; GTP, 0.2; EGTA, 1; Hepes buffer. The overall performance and linearity of


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the patch clamp, amplifiers, A-D board and computer system was checked before experimentation using a cell-model circuit to insure that the system reliably subtracted all linear components of capacitive and leakage current. The pH was adjusted with Hepes to 7.25. The electrode resistances ranged from 5–8 MΩ when filled with the cesium solution. The junction potential arising from the $Cs^+$ solution in the pipette and the 150 mM KCl agar reference electrode were nulled before patch formation.

Membrane currents were recorded using an Axopatch 1B patch clamp (Axon Instruments, Inc., Burlingame, CA). After cell-attached patch formation, the pipette and patch capacitance was nulled by analogue compensation. Upon obtaining the whole cell recording, the additional linear capacitive current, due to the charging and discharging of the cell membrane, was suppressed by a combination of the fast and slow time constant analogue capacitance compensation. Careful attention to this procedure was necessary to prevent amplifier saturation, and to minimize the time constant for the capacity current. The series resistance compensation was used in some experiments in which macroscopic $Na^+$ currents were recorded; in all cases the series resistance error was <2 mV.

The extracellular solutions contained (in millimolar) NaCl, 140; KCl, 5.4; $MgCl_2$, 1; $CaCl_2$, 1.8; glucose, 10; Hepes, 5. The pH was adjusted to 7.4. In experiments designed to examine nonlinear charge movement, all ionic currents were blocked. In addition to the internal $Cs^+$ solution (to block the early outward and delayed outward K currents), 10 mM Cs glutamate was added to the external solution (to block inward rectifier K currents), 10 μM tetrodotoxin was added to block the fast Na currents and $CoCl_2$ (3 mM) or $LaCl_3$ (100 μM) were added to block the Ca currents. In some experiments the Ca currents were not blocked completely to examine the charge movement and Ca current simultaneously.

In the experiments described and illustrated in this manuscript, a holding potential of −90 mV was employed to maximize the amount of $Ca^{++}$ channel charge movement, and to insure that an antagonistic effect of BAY K 8644 (which has been reported to occur at less negative holding potentials) was absent. The myocytes chosen for these experiments had little $I_{Na}$ and a reduced Na channel charge movement. The Na channel charge movement was much faster than the Ca channel charge movement, and they could be separated kinetically (Josephson, 1990, manuscript in preparation). Therefore, any residual Na channel charge movement would not obscure the effects of BAY K 8644 on the Ca channel charge movement, which is much slower.

*Data acquisition and analysis.* Data acquisition and analysis were done using the PCLAMP programs on an IBM AT computer. Membrane currents were filtered at 10 KHZ and amplified 10 times with an 8-pole Bessel filter, digitized at 30 μs/pt using a 12-bit A-D converter, and stored in 512 point files. To remove any residual linear capacitive and leakage current components, not nulled by the analogue suppression, a series of scaled "control" hyperpolarizing control steps (each 1/5 of the amplitude of the test step), was applied from a potential of −90 mV. This was followed by a 2-s duration holding potential (which could be from −120 to 0 mV). Then the voltage was briefly returned to the original −90 mV (for 1 ms) so that the test step would originate at the same potential, regardless of the holding potential.

After the test step there was a 2-s period at −90 mV before obtaining the subsequent control steps, to insure recovery of charge movement (or ionic current) that may have been immobilized (or inactivated) at a given holding potential. Individual sequences of test potentials were repeated and signals averaged 4–16 times to improve the signal-to-noise ratio. Whenever possible, the original voltage protocol was repeated at the end of an experiment, and the data was not analyzed if the linear capacity currents during the hyperpolarizing control steps were changed significantly.

The nonlinear charge at each test potential was measured by integrating the initial transient outward current (the ON charge) from the holding current baseline. Records in which a "pedestal" of current occurred (usually at positive potentials) were discarded from the analysis.

Experiments were conducted at 19–21°C. The following agents were added to the bath from concentrated stock solutions (stored at 4°C): tetrodotoxin (100 μM), $CoCl_2$ (300 mM), and BAY K 8644 ($10^{-4}$ M). Agents were dissolved in distilled water except for BAY K 8644, which was dissolved in 100% ethanol (final ethanol concentration of 0.1%). BAY K 8644 was stored in a light-tight container.

## RESULTS

Fig. 1 shows the effects of BAY K 8644 on both the gating currents, and the ionic currents carried by $Ca^{++}$ through cardiac (L-type) calcium channels. In this experiment the $Ca^{++}$ current was partially blocked (by 1 mM $Co^{++}$), in order to simultaneously record both the gating and the ionic currents. All other ionic and linear capacitive currents were blocked or subtracted, as described in Methods. The insets in *A* and *B* are diagrams of the test voltage steps. The five superimposed currents were elicited by the test steps to −20 through +20 mV, in 10-mV increments. The holding potential was chosen to be −90 mV, in order to elicit a pure agonist effect of BAY K 8644 (3). *A* shows the control (ON) gating currents (transient outward current), followed by the activation of the inward $Ca^{++}$ currents. Upon repolarization of each voltage step, there is an inward tail current, composed of both the $Ca^{++}$ tail current and the OFF (deactivation) charge

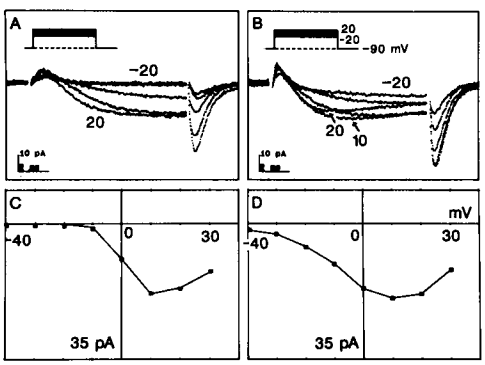


FIGURE 1 BAY K 8644 shifts both the $Ca^{++}$ channel gating current and the $Ca^{++}$ current recorded from an embryonic chick ventricular myocyte. (*A*) Control currents. (*B*) Currents recorded 1 min after the addition of BAY K 8644 (1 μM). Each current trace is the average of four repetitions. The test voltage protocol is shown in the insert; the currents recorded at −20 to +20 mV (in 10-mV increments) are displayed (superimposed) in the figure (as labeled). The holding potential was −90 mV (*dotted line in protocol*). The calcium current was suppressed, but not blocked by 1 mM $Co^{++}$. *C* and *D* are the current-voltage relationships for the peak inward $Ca^{++}$ currents recorded for control (*C*) and BAY K 8644-treated (*D*) currents.

movement. The current-voltage relationship for the peak $Ca^{++}$ current measured during the voltage step, is given in *C*. The control inward $Ca^{++}$ current activated at potentials $> -30$ mV, and reached a maximum at $+10$ to $+20$ mV.

Fig. 1 *B* shows the changes in the gating currents and $Ca^{++}$ currents 1 min after exposure to BAY K 8644 (1 $\mu$M). The most striking effect on the ON gating currents was a shift in their time course, so that they peaked earlier than the control gating currents. This shift in the gating currents was paralleled by the following changes in the macroscopic $Ca^{++}$ currents.

First, there was a negative shift in the voltage-dependence for activation of the calcium current after BAY K 8644. As shown in Fig. 1 *D*, the $Ca^{++}$ current activated at a potential 20 mV more negative in the presence of BAY K 8644, as compared to the control current. This resulted in a voltage-dependent enhancement of $I_{Ca}$. (In this experiment, the maximum peak $I_{Ca}$ was not increased due to a rundown of $I_{Ca}$.) Second, the time course of the $Ca^{++}$ currents was changed, so that they activated and inactivated faster in the presence of BAY K 8644. Similar effects on the gating and calcium currents (shifts in $I_{Ca}$ activation of $-12$ to $-17$ mV) were observed in three additional experiments.

To further characterize the changes in the kinetics of the gating currents by BAY K 8644, and to be able to correlate those with the changes observed in the $Ca^{++}$ currents, subsequent experiments were conducted following greater suppression of the $Ca^{++}$ currents. The nature and identity of the charge movement signal was established by the application of the following criteria (12). Integration of the control charge movement, after blockade of all ionic currents, demonstrated that there is a close agreement between the amount of gating charge moved during depolarization (ON) and repolarization (OFF). This test substantiates that the signal is capacitive in nature. The voltage-dependence for the equilibrium ON ($Q_{ON}$) and OFF ($-Q_{OFF}$) charge movement is a nonlinear function of potential, which saturates with increasing depolarization.

In addition, several other key characteristics of the nonlinear charge movement strongly suggest that it is involved with the voltage-dependent activation of $Ca^{++}$ channels. First, the charge movement precedes significant activation of the $Ca^{++}$ current. Second, the voltage-dependence for the activation of charge movement occurs over a similar range of potentials as for the activation of $I_{Ca}$. Third, the amount of charge that moves during a test depolarization can be reduced (immobilized) by the steady-state application of a less negative holding potential, which also inactivates $I_{Ca}$. Fourth, the amount of charge moved is appropriate for the size of $I_{Ca}$ ($\sim 100 >$ pA) and the surface area ($\sim 200$ $\mu m^2$) of these myocytes. If it is estimated that there are 5–10 channels per $\mu m^2$, and that each channel contributes a gating charge of $4e^-$, then the approximate charge movement is 0.3 nC/$\mu$F. This value is within a factor of 2–3 of the experimental measurement of gating charge movement. In addition, two L-type $Ca^{++}$ channel modulators, isoproterenol (12), and BAY K 8644 (present manuscript) act on the charge movement in a manner which correlates with the effects of these agents on $I_{Ca}$.

Before the addition of BAY K 8644, a control period of 5–10 min was observed to insure that the charge movement did not change with time. During this period, no alterations were made to the capacity or series resistance compensations. This procedure ruled out the possibility that small changes in the access resistance to the cell might have occurred which could affect the time course of the charge movement.

It should be noted, however, that an increase in the access resistance (which does occasionally develop during an experiment) would lead to a reduction of the clamp speed and, therefore, an apparent slowing of charge movement. This is clearly opposite to the effects of BAY K 8644, and cannot, therefore, provide an alternative explanation for the results.

Fig. 2 shows the isolated $Ca^{++}$ channel gating currents recorded at test potentials of $-10$ mV (*A*), and $+20$ mV (*B*). The traces labeled "CONT" are the control gating currents, and those labeled "BAY K" are the currents 1 min after exposure to BAY K 8644 ($10^{-6}$ M). At all test potentials there was a clear shift of the peak of the gating current to earlier times. The effect of the shift on the cumulative charge movement is demonstrated by the charge integral of each of the gating currents shown in (*A*) and (*B*), which are displayed above. Although the total charge moved in the steady-state is similar in the absence and presence of BAY K 8644, a greater fraction

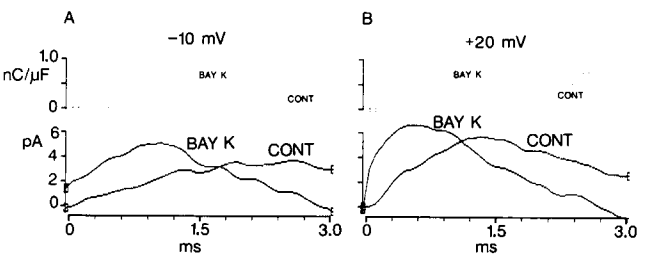


FIGURE 2 BAY K 8644 shifts the time course of the $Ca^{++}$ channel gating currents. *A* shows the gating currents (*lower traces*) during control (CONT) and 1 min after BAY K 8644 ($10^{-6}$), and (*above*) the charge integrals of those currents (*points*), at a test potential of $-10$ mV. *B* shows the gating currents and charge integrals (points) at a test potential of $+20$ mV. The holding potential was $-90$ mV. The cell capacitance was 7 pF.

of the charge moves earlier with BAY K 8644. The faster activation kinetics results in the leftward shift of the charge-integral.

The voltage-dependence of the time to the peak (Tp) of the ON gating current, under control conditions (*open circles*) and after BAY K 8644 (*solid circles*), is shown in Fig. 3 *A*. The gating currents tend to display a biphasic voltage dependence; they peak later in time over the range of potentials between −50 to −30 mV, and peak faster with increasing depolarization between −30 and +30 mV. It can be seen that BAY K 8644 shortened the time to peak of the gating currents at all potentials tested. These results were confirmed in four other experiments with BAY K 8644, in which Tp was shortened by 0.5 to 1.0 ms.

As shown in Fig. 1 and 2, BAY K 8644 caused the $Ca^{++}$ channel gating charge to move earlier during a depolarization. To quantitate the amount of charge moved before the peak of $I_{Ca}$, isochronal integration of the gating currents was performed. The voltage dependence of the charge movement for the control, and BAY K 8644-treated currents is shown in Fig. 3 *B*. BAY K 8644 produced a shift of −12 mV (as measured at 50% of the total charge). Comparable shifts (−7 to −13 mV) were obtained in four additional experiments.

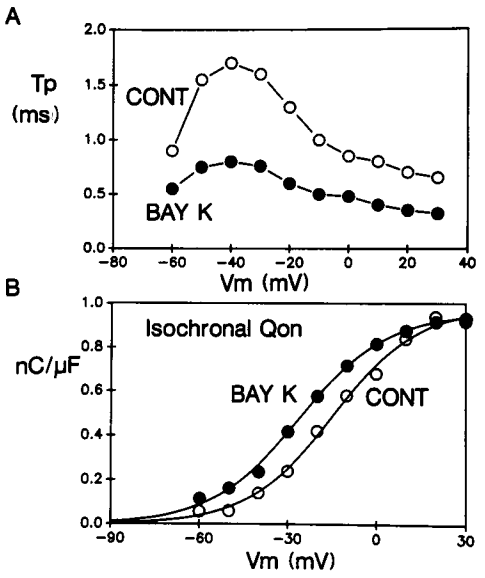


FIGURE 3 BAY K 8644 shifts the time to peak (*A*), and the isochronal charge movement (*B*) of the $Ca^{++}$ channel gating currents. In *A*, the time to peak ($T_p$) of the control (*open circles*), and the BAY K 8644-treated (*solid circles*) gating currents are plotted as a function of the test potential. In *B* the gating currents before (*open circles*) and after $10^{-6}$ BAY K 8644 (*closed circles*) were integrated isochronally (i.e., during the initial 1.5 ms of depolarization) and plotted as a function of the test potential. The cell capacitance was 6 pF.

## DISCUSSION

BAY K 8644 did not change the total charge moved at any test potential (steady-state measurement), which suggests that the total number of $Ca^{++}$ channels is not altered by this agent. Instead, it appears that the kinetics of charge movement are more rapid after the binding of BAY K 8644 to the $Ca^{++}$ channel. The result is that a greater fraction of the gating charge is moved (i.e., more $Ca^{++}$ channels undergo voltage-dependent activation) earlier during the depolarization.

The voltage-dependent, negative shift of the activation of $I_{Ca}$ after exposure to BAY K 8644 has also been reported previously for a variety of excitable tissues (3–8). It seems likely that the negative shift in the $Ca^{++}$ channel gating current, described above, underlies the negative shift of the activation of the macroscopic $I_{Ca}$. Furthermore, the more rapid activation kinetics of $I_{Ca}$ in the presence of BAY K 8644 (detected as a decrease in latency of first opening in single-channel experiments) may also be the result of the more rapid kinetics of $Ca^{++}$ channel charge movement.

How does the binding of BAY K 8644 to the $Ca^{++}$ channel alter the gating charge movement? Although BAY K 8644 does not appear to enhance intracellular levels of cyclic AMP (11), it is intriguing to speculate that one possible action of this agent may be to prevent the dephosphorylation of the $Ca^{++}$ channel. β-Adrenergic stimulation (by isoproterenol), or the addition of 8-br-cyclic AMP has been shown to have effects similar to BAY K 8644 on the $Ca^{++}$ channel gating currents (12) and on the single $Ca^{++}$ channel currents (13). These agents presumably act through a cyclic AMP-dependent phosphorylation of the $\alpha_1$ subunit of the $Ca^{++}$ channel. BAY K 8644 may induce a conformational change in the $Ca^{++}$ channel structure which either limits the interaction of the endogenous phosphatase with the phosphorylation site, or mimics the conformational change induced by the phosphorylation of the subunit. Although they are attractive, these hypotheses cannot account for the dissimilarities between the effects of BAY K 8644 and the putative phosphorylation of the $Ca^{++}$ channel. For example, BAY K 8644 increases, but β-adrenergic stimulation decreases the rate of inactivation of $I_{Ca}$ (15). Furthermore, the enhancement of $I_{Ca}$ by BAY K 8644 has been shown to be additive with the increase produced by β-adrenergic stimulation, suggesting different sites of action for these two agents (15).

In conclusion, the present report provides an explanation for the negative shift and more rapid kinetics of $I_{Ca}$ observed in the presence of the dihydropyridine agonist, BAY K 8644. It seems likely that, in addition to BAY K

8644 and isoproterenol (12), many other channel regulatory agents may be found to produce their effects through changes in the gating charge movement which governs the opening of voltage-dependent ion channels.

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