# Early Afterdepolarizations: Mechanism of Induction and Block

## A Role for L-Type $Ca^{2+}$ Current

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**Early afterdepolarizations (EADs) are a type of triggered activity found in heart muscle. We used voltage-clamped sheep cardiac Purkinje fibers to examine the mechanism underlying EADs induced near action potential plateau voltages with the $Ca^{2+}$ current agonist Bay K 8644 and the effect of several interventions known to suppress or enhance these EADs. Bay K 8644 produced an inward shift of the steady-state current-voltage relation near plateau voltages. Tetrodotoxin, lidocaine, verapamil, nitrendipine, and raising $[K]_o$ abolish EADs and shift the steady-state current-voltage relations outwardly. Using a two-pulse voltage-clamp protocol, an inward current transient was present at voltages where EADs were induced. The voltage-dependence of availability of the inward current transient and of EAD induction were similar. The time-dependence of recovery from inactivation of the inward current transient and of EAD amplitude were nearly identical. Without recovery of the inward current transient, EADs could not be elicited. The inward current transient was enhanced with Bay K 8644 and blocked by nitrendipine, but was not abolished by tetrodotoxin or replacement of $[Na]_o$ with an impermeant cation. These results support a hypothesis that the induction of EADs near action potential plateau voltages requires 1) a conditioning phase controlled by the sum of membrane currents present near the action potential plateau and characterized by lengthening and flattening of the plateau within a voltage range where, 2) recovery from inactivation and reactivation of L-type $Ca^{2+}$ channels to carry the depolarizing charge can occur. Our results suggest an essential role for the L-type $Ca^{2+}$ "window" current and provide a framework for understanding the role of several membrane currents in the induction and block of EADs. (*Circulation Research* 1989;64:977–990)**

Early afterdepolarizations (EADs) are a type of triggered activity that can arise in heart muscle before action potential repolarization is completed.[1] An increasing number of interventions affecting several cellular mechanisms have been shown to induce or suppress EADs (for brief review, see Damiano and Rosen[2] and January et al[3]). Consequently, several cellular mechanisms have been implicated in the generation of EADs, including $Na^+$ "window" or slowly inactivating current,[4] $Ca^{2+}$ current,[5,6] $i_{Xl}$,[7] the transient inward current $i_{TI}$ activated by elevated intracellular $Ca^{2+}$,[8] the Na-Ca exchange mechanism,[9] and intercellular $K^+$ accumulation.[4] The interpretation of these results is complicated, thus the precise mechanism(s) for the induction and block of EADs remains unknown. Adding to this uncertainty is the observation that EADs may arise from two voltage ranges.[2]

Recently, we reported a model for inducing EADs in sheep and canine cardiac Purkinje fibers using the $Ca^{2+}$ current agonist Bay K 8644.[3] Bay K 8644 produced concentration- and frequency-dependent lengthening of action potential duration and flattening of the plateau. At low stimulation frequencies, EADs were induced from an average take-off potential of −34 mV. Several drug and ionic interventions that affect different membrane currents suppress or enhance the induction of EADs with Bay K 8644, and these observations were similar to results obtained in other experimental EAD models (e.g., $Cs^+$, quinidine, and low $[K]_o$). The EAD peak voltage also showed marked dependence on its take-off potential. We interpreted these results to suggest that EAD induction required two phases: 1) an initiation or conditioning phase controlled by the sum of membrane currents present at the action potential plateau and characterized by its lengthen-

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Supported in part by Grant HL-38927 from the National Institutes of Health, Bethesda, Maryland, and a grant from Marquette Electronics Inc, Milwaukee, Wisconsin.

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Received May 3, 1988; accepted October 24, 1988.

ing and flattening within a voltage range where, 2) there could occur recovery from inactivation and reactivation of an inward current possibly carried by L-type $Ca^{2+}$ channels.

This report presents experiments that test this hypothesis. Our results suggest an essential role for L-type $Ca^{2+}$ channels in the depolarization of EADs and provide a framework for understanding the roles of several membrane currents in the induction and block of EADs. Preliminary reports of these results have appeared.[10,11]

## Materials and Methods

### *Preparations*

Short segments of 0.8–2.0 mm length and 60–250 $\mu$m diameter were isolated from free-running sheep Purkinje fibers. The preparations were obtained as previously described.[3] Adequate space clamp conditions should exist in these short segments.

### *Electrical Recordings*

The experimental technique was similar to that used previously. Briefly, each short segment was mounted in a Lucite chamber (volume 1.5 ml) and impaled with two 3 M KCl–filled glass microelectrodes. The current-passing microelectrode was lightly dry bevelled or stored overnight to improve its current-passing characteristics and was inserted near the center of the preparation. The voltage-measuring microelectrode was inserted nearby within 200 $\mu$m. Fibers were voltage-clamped using a two-microelectrode technique[12] (control amplifier model 3584, Burr-Brown, Tucson, Arizona). Command pulses were generated by a digital timing network (World Precision Instruments, New Haven, Connecticut). Tension was measured with an isometric dual capacitance transducer (Cambridge Technology, Inc, Cambridge, Massachusetts). Data were recorded on FM tape for playback as previously described. When needed, EADs were sampled in 12-bit resolution at 1 kHz and dV/dt was determined digitally on a laboratory computer.

### *Solutions*

Tyrode's solution contained (mM) NaCl 127, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.05, $NaH_2PO_4$ 2.4, $NaHCO_3$ 22, and glucose 5.5. For $Na^+$-free Tyrode's solution, NaCl, $NaHCO_3$, and $NaH_2PO_4$ were replaced by 139 mM tetramethylammonium chloride, 10 mM HEPES, with the pH adjusted to 7.4 by tetramethylammonium hydroxide. Solutions were continuously gassed with 95% $O_2$-5% $CO_2$, and the temperature in the chamber was 37±0.5° C. The flow rate was 3–5 ml/min.

The Bay K 8644 concentration used in all experiments was $1\times10^{-6}$ M (Bay K 8644-Tyrode's solution). Tetrodotoxin (TTX), lidocaine, nitrendipine, and verapamil were added to Bay K 8644-Tyrode's solution as required. The compositions of the drug stock solutions were given previously.[3] When used, $[K]_o$ was lowered to 3.5 mM without osmotic correction.

### *Experimental Protocols*

Purkinje fiber short segments were equilibrated by stimulation at 1 Hz for 1 hour. The stimulation frequency was then changed to 0.1 or 0.2 Hz. Our protocols required that every fiber have normal resting and action potentials and be free of automaticity and afterdepolarizations before initiating an experiment.

Two voltage-clamp protocols were used. The first was designed to study the membrane currents present near action potential plateau voltages. This steady-state membrane current was measured at the end of 800-msec long steps to different voltages or in response to a slowly rising (4 mV/sec) voltage ramp. Both methods gave identical results except at voltages positive to those where the transient outward current became large (see Fozzard and Hiraoka[13]). Positive to about −20 to −10 mV, the steady-state outward current was slightly greater when measured at the end of square steps. For individual experiments, results were obtained using only one method.

We also used a two-pulse voltage-clamp protocol to study the recovery from inactivation of an inward current transient that underlies the depolarization of EADs. A schematic representation of this protocol is shown in Figure 1 (upper trace). From the holding potential, a 200-msec long depolarizing step to +10 to +15 mV was applied to activate $Ca^{2+}$ current. At these voltages with Bay K 8644 present, $Ca^{2+}$ channel activation is nearly maximal.[14,15] This was followed by a conditioning step to a voltage near the take-off potential of EADs. A second depolarizing (test) step was then applied and could elicit an inward current transient. By varying the duration or voltage of the conditioning step, recovery from inactivation of the inward current transient was studied. By varying the amplitude of the test step, voltage-dependent properties of this inward current transient also were studied. A recording of membrane current obtained with this protocol is shown in Figure 1 (lower trace, Bay K 8644-Tyrode's solution). From the holding potential of −50 mV, the initial step to +9 mV elicited an inward $Ca^{2+}$ current that had decayed by the end of the step. The conditioning step to −29.5 mV (the EAD take-off potential in this fiber) was 500 msec duration. With the test step to −17 mV, a net inward current transient was obtained (arrow, lower trace). EADs also were elicited with this protocol by switching out the voltage clamp feedback loop at the end of the conditioning step (release from voltage clamp, arrow in upper trace). By varying the duration or voltage of the conditioning step before release from voltage clamp, time- and voltage-dependent properties of EADs were studied. Thus, the same initiating conditions could be used to

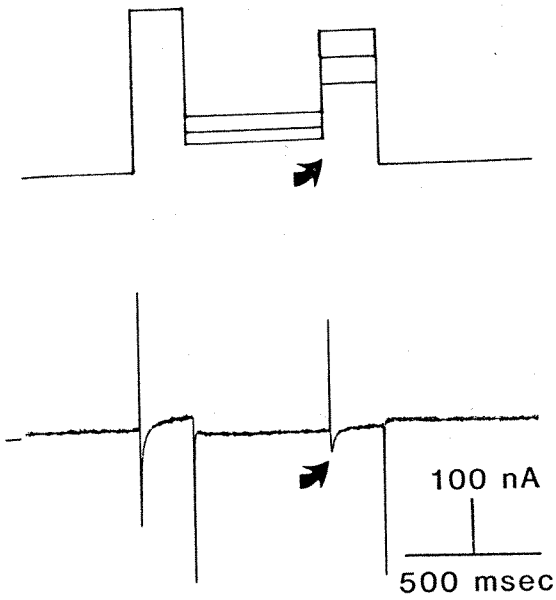



FIGURE 1. *The two-pulse voltage-clamp protocol used to study recovery of early afterdepolarizations (EADs) and an inward current. From the holding potential (upper trace), a large amplitude depolarizing step was followed by a conditioning step to voltages near the EAD take-off potential. A second depolarizing (test) step was then applied (arrow) to elicit an inward current transient, or the preparation was released from voltage clamp to elicit EADs. Time- and voltage-dependent properties of the recovery of the inward current transient or the EADs were studied by varying the duration or amplitude of the conditioning step. Voltage-dependence of the inward current transient was studied by varying the amplitude of the test step. A membrane current record (lower trace, dash=0 nA) shows the inward current transient obtained with this protocol (arrow).*

study EADs and their underlying currents in the same fiber.

We used holding potentials of −50 mV, which voltage inactivated the $Na^+$ channel current, or −75 mV, which was close to the resting potential. At these holding potentials, Bay K 8644 is a $Ca^{2+}$ current agonist.[14,16,17] Stimuli or depolarizing step sequences were applied at a constant rate of 0.1 or 0.2 Hz to avoid the frequency-dependent effects of the Bay K 8644.[14] Our previous studies have shown that most Purkinje fibers exposed to $1\times10^{-6}$ M Bay K 8644 and stimulated at 0.2 Hz develop EADs.[3]

In most experiments, the Purkinje fibers were exposed to normal Tyrode's solution. Although the presence of several ionic species complicates the interpretation of the underlying currents, this approach was necessary because of the number of membrane currents postulated to participate in EADs. In some experiments, we used ion substitution or drug block to identify specific currents. We did not attempt to correct raw membrane currents for contamination by other currents, rather our interest was in the net membrane current.

Data for recovery experiments were analyzed using single-exponential curve fitting and least-squares regression analysis. A value of $p<0.05$ was considered statistically significant.

## Results

### *Effect of Bay K 8644 on the Steady-State Current-Voltage Relation*

Previous studies in multi- and single-celled preparations have shown that Bay K 8644 augments the peak inward current carried through $Ca^{2+}$ channels and that the peak current-voltage relation is shifted negatively.[14,15,17,18] We obtained similar results in our experiments as shown in Figure 2 ($n=2$). The control (Tyrode's solution) current-voltage plot shows the peak inward current obtained with depolarizing steps from the holding potential of −50 mV. After approximately 10 minutes' exposure to Bay K 8644-Tyrode's solution, the amplitude of the inward current was markedly enhanced, and the peak of the current-voltage relation was shifted negatively. Current and voltage traces in Bay K 8644-Tyrode's solution are shown in the inset.

Bay K 8644 increases action potential duration and flattens the plateau[3,18] before inducing EADs, suggesting that Bay K 8644 alters the steady-state current near action potential plateau voltages. Records of membrane currents for control conditions (Tyrode's solution) and after Bay K 8644 exposure obtained using voltage ramps are shown at the top of Figure 3A, and the membrane currents are plotted together below. For control conditions, the N-shaped steady-state current-voltage relation was outward at all voltages positive to the resting potential of −72 mV. After exposure to Bay K 8644-Tyrode's solution had resulted in EADs, the steady-state current-voltage relation was redetermined. Bay K 8644 produced an inward shift in the steady-state current-voltage relation near action potential plateau voltages, and in this fiber the current became net inward for voltages between −40 and −28 mV. At more negative voltages Bay K 8644 had no effect, whereas at more positive voltages the current was slightly less outward. The current-voltage relation had three zero current intercepts, two having a positive slope. This may suggest the presence of two stable resting potentials[19]; however, most fibers exposed to Bay K 8644 repolarized spontaneously[3] and in only a few fibers repolarization occurred after passage of small repolarizing current pulses. Figure 3B shows similar results from a different fiber studied with 800-msec long depolarizing square steps. Bay K 8644 shifted the steady-state current-voltage relation inwardly near the EAD take-off potential, whereas, for control conditions in Tyrode's solution, the steady-state current had been outward at all voltages positive to the resting potential (control records not shown). The inward shift induced with Bay K 8644 decayed slowly (see third panel, voltage step to −30 mV) possibly

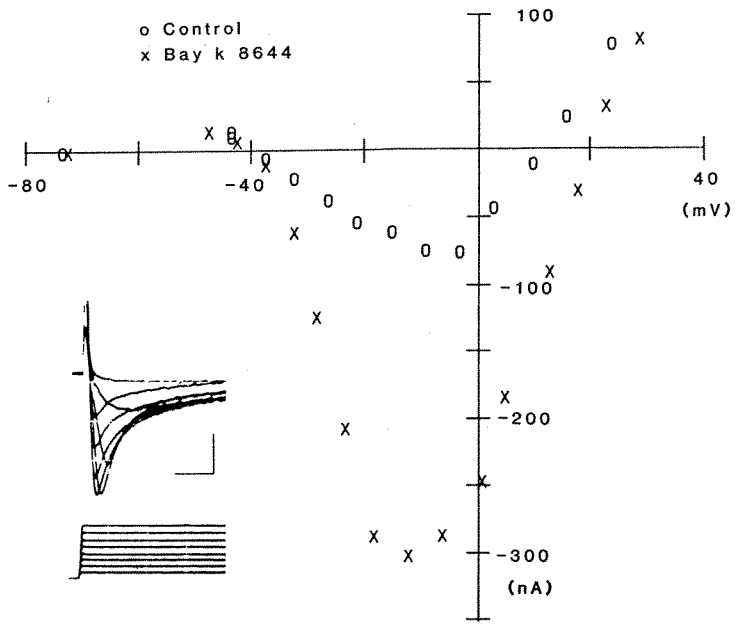



FIGURE 2. *Increased inward current with Bay K 8644. From a holding potential of −50 mV, $Ca^{2+}$ currents were elicited with step depolarizations for control (Tyrode's solution) and with Bay K 8644 ($1\times10^{-6}$ M). The peak current-voltage relations show that Bay K 8644 markedly increased inward current and shifted the peak negatively. Records of membrane currents with Bay K 8644 are shown in the inset (horizontal calibration=20 msec, vertical calibration=50 mV and 100 nA).*

leading to repolarization. Figure 3B also shows that an oscillatory current was not present during the depolarizing step or after its termination. Even with current resolutions of <1 nA, no oscillatory ($i_{Ti}$-like) current was observed ($n$=14). Bay K 8644 consistently produced an inward shift in the steady-state current-voltage relation near action potential plateau voltages in 25 Purkinje fiber short segments.

*Effect of TTX, lidocaine, verapamil, nitrendipine, and $[K]_o$ on the steady-state current.* EADs induced with Bay K 8644 can be suppressed with interventions that shorten action potential duration by different mechanisms.[3] We studied the effect of several interventions on the Bay K 8644–induced changes in the steady-state current-voltage relation as is shown in Figure 4. Bay K 8644 was continuously present in the superfusate. In each panel, the first current-voltage plot (Bay K 8644) shows the inward shift at plateau voltages produced by Bay K 8644. The addition of TTX (upper left panel), lidocaine (upper right panel), nitrendipine (lower left panel), and verapamil (lower right panel) reduced the inward shift (Bay K 8644 plus drug). Washout of each intervention resulted in the return to the initial conditions (Bay K 8644 minus drug). Similar findings were observed in four experiments with TTX, two experiments with lidocaine, four experiments with verapamil, and five experiments with nitrendipine. Action potential duration and induction of EADs depends on $[K]_o$,[3,20] and small decreases in $[K]_o$ shift the steady-state current-voltage relation inwardly near plateau voltages (for example, see Isenberg[21]). We confirmed this finding in our model (Bay K 8644-Tyrode's solution) when $[K]_o$ was lowered from 5.4 and 3.5 mM, which was reversed by restoring $[K]_o$ to 5.4 mM ($n$=3, data not shown).

### *An Inward Current Transient Underlies Depolarization of EADs*

Although a shift in the steady-state current-voltage relation can account for changes in action potential duration and plateau voltage, these effects do not explain EAD depolarization for which an additional net inward current is required. We have hypothesized that slowing of action potential repolarization could lead to the time- and voltage-dependent recovery of an inward current. Figure 5 shows records from one fiber that illustrate the protocol used to elicit EADs and to identify an inward current transient that underlies it. Bay K 8644 was continuously present. In panel A an action potential triggered an EAD from a take-off potential of −33 mV. EADs also could be elicited by releasing the fiber from voltage clamp as shown in panels B and C. After the initial 200-msec long depolarizing step to +12 mV, a conditioning step to −32 mV (near the EAD take-off potential) was applied. Releasing the preparation from voltage clamp 500 msec later (arrows in current traces) resulted in EADs. At the same conditioning voltage where EADs were obtained, inclusion of the test step (two-pulse voltage-clamp protocol) elicited an inward current transient (arrows, panels D and E). The amplitude of the EADs or the inward current transient was virtually unchanged by holding potentials of −75 mV (panels B and D) or −50 mV (panels C and E).

*Voltage-dependence of EADs and the inward current transient.* We previously showed that the EAD peak voltage varied inversely with the take-off potential and that with sufficient depolarization the take-off potential and peak voltage became equal (i.e., no EADs). Therefore, we studied the voltage-dependence of EADs and the inward current tran-

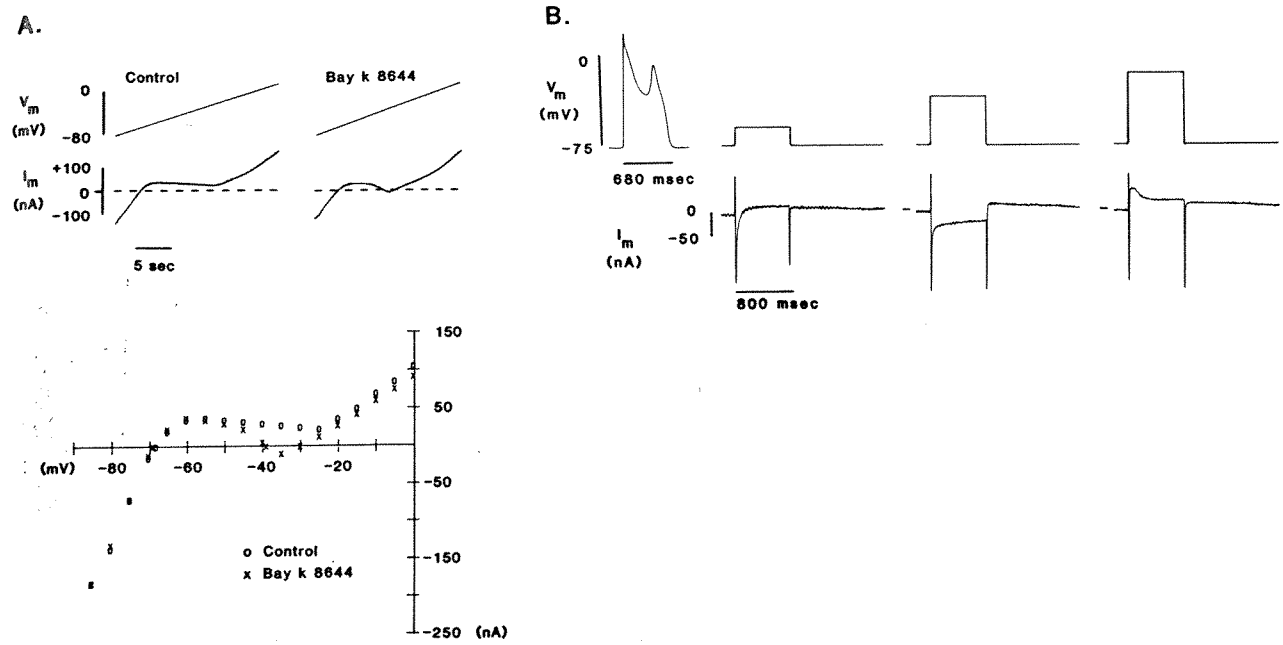



FIGURE 3. *Steady-state current and Bay K 8644.* A: *Steady-state currents for control (Tyrode's solution) and with Bay K 8644 ($1\times10^{-6}$ M) were obtained using voltage ramps. Original records are shown above and data points at 5 mV increments are plotted together below. Bay K 8644 produced an inward shift in the steady-state current-voltage relation near action potential plateau voltages. Dashed line=0 nA.* B: *An EAD was triggered with each action potential in a different fiber in Bay K 8644-Tyrode's solution. Voltage clamp steps then were applied from −75 mV. Steps to −55, −30, and −9 mV are shown and elicited large inward ($Na^+$ and $Ca^{2+}$) currents. At the end of the step to −30 mV, membrane current was inward and had decayed to a value of −23 nA, whereas, for the steps to −55 or −9 mV, the steady-state current at the end of the depolarization was outward (+7 and +13 nA, respectively). Also shown is the absence of an oscillatory ($i_{TI}$-like) current.*

sient using the two-pulse voltage-clamp protocol. Records of EADs elicited using the release from voltage clamp protocol are shown in Figure 6A (inset). From the holding potential, the initial depolarizing step was followed by a 600-msec long conditioning step to different voltages. The duration of conditioning step was adequate to permit the recovery from inactivation of the inward current transient. Release from voltage clamp initiated EADs whose peak voltage varied inversely with the conditioning voltage as shown by the plot in panel A. As the conditioning voltage became less negative, the EAD peak voltage decreased until, at approximately −27 mV, no EAD was obtained. As the conditioning voltage became more negative, EAD peak voltage increased to a maximum in this fiber of +12 mV, which was obtained from a conditioning voltage of −42 mV. Release from more negative conditioning voltages resulted in the rapid membrane repolarization to the resting potential with no EADs elicited. The dependence of EAD peak voltage on the conditioning voltage was unchanged at holding potentials of −70 mV (filled circles) or −50 mV (filled squares). Figure 6B shows results obtained using the two-pulse voltage-clamp protocol in the same fiber (see inset, holding potential −50 mV). These current-voltage plots of the peak inward current transient were determined by varying the amplitude of the test step, and each plot was obtained from a different conditioning voltage (−40, −34, −30, −27, and −24 mV) over the range where EADs occurred. The inward current transient amplitude decreased as the conditioning voltage became more positive. At the conditioning voltage of −24 mV, the current-voltage relation was net outward except for near its peak. At a conditioning voltage of −27 mV, the smallest amplitude depolarizing steps also failed to elicit a net inward current. This conditioning voltage was nearly identical to the conditioning voltage at which the EAD amplitude approached zero. The steady-state current-voltage relation also is plotted (dashed line). The region of its negative slope intercepts the zero current axis at approximately −43 mV, which is near the conditioning voltage below which EADs were not elicited with the release from voltage-clamp protocol.

These findings show that the inward current transient and the EAD peak voltage are steeply voltage-dependent over similar conditioning voltage ranges. Moreover, the conditioning voltage at which the EAD amplitude approached zero (i.e., no EAD) was nearly identical to the conditioning voltage where the peak inward current transient remained net outward (i.e., no depolarizing current). Finally, the region of negative slope of the steady-state current-voltage plot intercepted the zero current

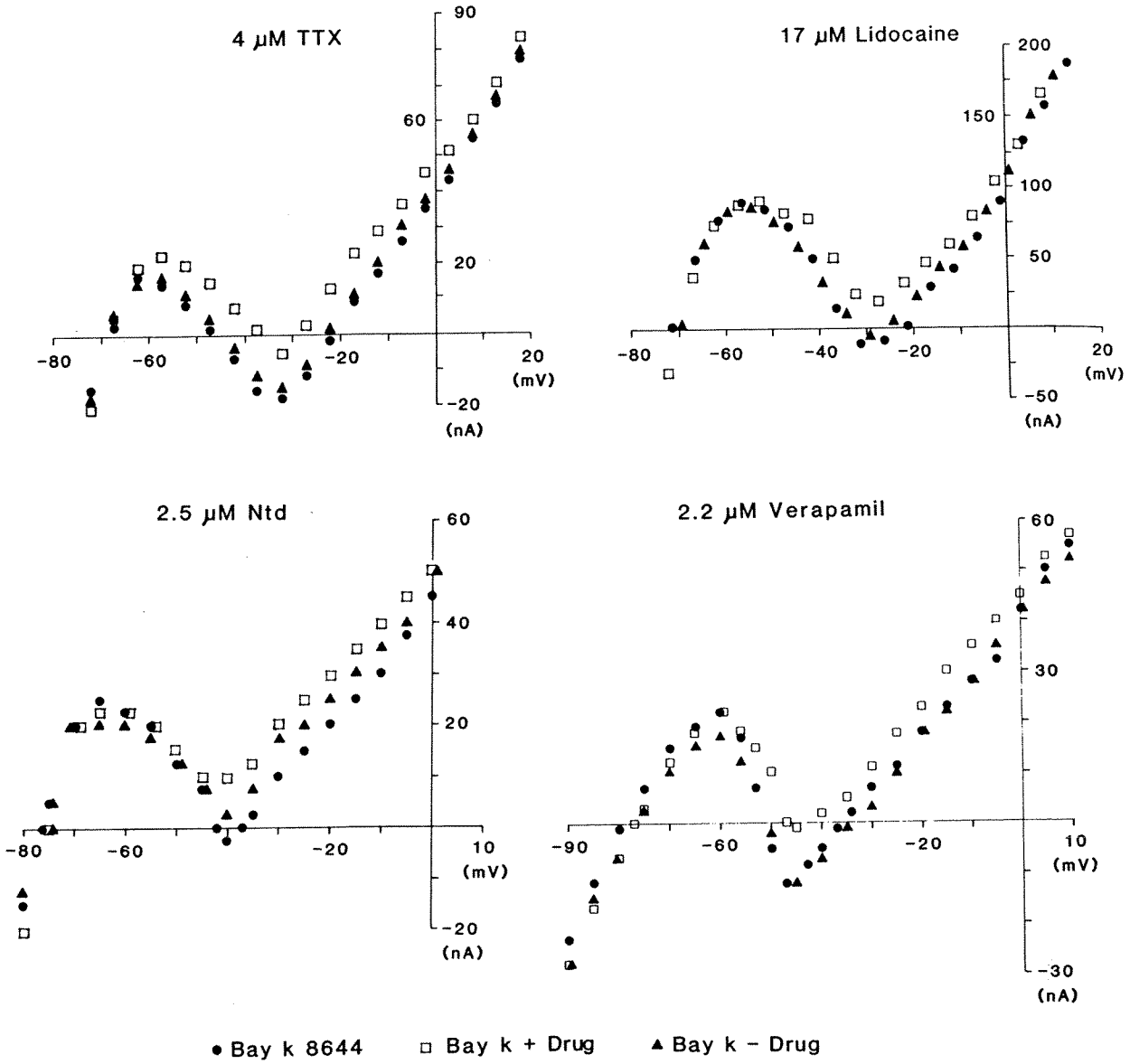
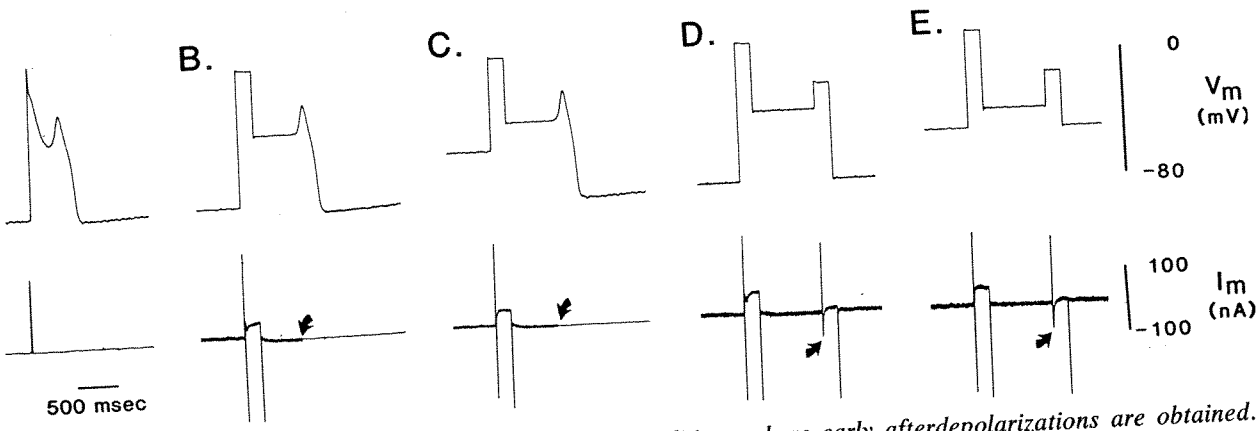



FIGURE 4. *The effect of tetrodotoxin (TTX, $4\times10^{-6}$ M), lidocaine ($1.7\times10^{-5}$ M), nitrendipine (Ntd, $2.5\times10^{-6}$ M) and verapamil ($2.2\times10^{-6}$ M) on the steady-state current-voltage relation in the presence of Bay K 8644. The first plot in each panel (●), obtained after early afterdepolarizations were induced, shows the typical Bay K 8644 effect on the steady-state current-voltage relation. The second plot in each panel (□) was obtained following block of early afterdepolarizations by the indicated drug. The third plot was obtained after washout of the drug (▲). Each drug reversibly shifted the steady-state current-voltage relation outwardly near action potential plateau voltages.*

axis close to the most negative conditioning voltage from which EADs were elicited. We obtained similar findings in three additional fibers.

*Time-dependence of recovery of EADs and the inward current transient.* Experimental records of the time-dependence of recovery of EADs and the inward current transient are shown in Figure 7. EADs were initiated using the release from voltage-clamp protocol with the duration of the conditioning voltage step to −30 mV varied between 0 and 800 msec (panel A). Lengthening the interval of the conditioning step resulted in EADs of increasing amplitude ($V_{EAD}$=EAD peak voltage−conditioning voltage) to a maximum value, and this time-dependence of recovery of EAD amplitude is plotted below the example records. Recovery from inactivation of the inward current transient was studied in the same fiber using the two-pulse voltage-clamp protocol (panel B). Lengthening the interval of the conditioning step caused the inward current transient amplitude to increase until a maximum value was obtained, and this time-dependence of recovery from inactivation of the peak inward current transient is plotted below the example records. These measurements of the time-dependence of recovery of EAD amplitude and of the peak inward




GURE 5. *An inward current transient is present under conditions where early afterdepolarizations are obtained. ecords are shown of membrane voltage ($V_m$, upper traces) and current ($I_m$, lower traces) from the same fiber ntinuously exposed to Bay K 8644. With each action potential an early afterdepolarization was triggered from a ke-off potential of −33 mV* (panel A). *Similar early afterdepolarizations were induced when the fiber was released from oltage clamp (arrows in* panels B and C, *conditioning voltage −32 mV). With the two-pulse voltage-clamp protocol, an ward current transient was present (arrows in* panels D and E). *Similar results were obtained from holding potentials f −75 mV (panels B and D) or −50 mV (panels C and E).*

current transient were then normalized with the nitial value (0 mV amplitude or 0 nA) as 0% and the naximum value as 100%. These data were replotted ogether against the conditioning step recovery interval (panel C) and show that the time courses of recovery for EAD amplitude and for the peak inward current transient were similar.

Results from five fibers where both the time course of recovery of EAD amplitude and of the peak inward current transient were measured in the same fiber are shown in Figure 8A. For each fiber, the conditioning voltage was close to the EAD take-off potential. These data show that recovery of EAD amplitude and of the peak inward current transient in these fibers had similar time dependence. To test for correlation, the data were transformed by plotting the percent recovery of EAD amplitude against the percent recovery of the peak inward current transient obtained for the same recovery interval (Figure 8B). Linear regression analysis revealed a highly significant correlation ($r$=0.84, $p$<0.001) between the recovery of EAD amplitude and the recovery of the amplitude of the peak inward current transient.

The interpretation of these data may be complicated by several factors. One is latency between the development of the inward current transient and the EAD. The peak of the inward current transient is reached a few milliseconds after application of a depolarizing step. In contrast, following release from the conditioning step, EAD depolarization requires more time to reach a peak value. Latency in the development of the EAD was present over the range of recovery intervals we studied but was greatest at short intervals. Because the EAD peak voltage is reached after a longer delay, time- and voltage-dependent changes may occur in the underlying membrane currents. To account for possible effects of latency, we measured the recovery interval for EADs two other ways: first, as the time from the onset of the conditioning step to the maximum dV/dt of the EAD, and second, as the time required for the EAD to reach its peak voltage. The major effect of different recovery interval measurement methods was to shift the EAD recovery curve along its time axis. When these results from the five fibers were treated the same as the data in Figure 8B, each type of EAD recovery interval analysis gave a slightly different linear regression relationship (Table 1). Regardless of the EAD recovery interval used, we obtained a highly significant correlation ($p$<0.001) between the recovery of EAD amplitude and the recovery of the amplitude of the peak inward current transient. A second possible source of error is the method used to measure the inward current transient. We made no attempt to correct it for contamination by other membrane currents; rather, we measured absolute current values. Although attempts to correct the inward current transient for background and outward current contamination might alter slightly the peak values and the regression relations, it is unlikely to alter the conclusions from our findings. Lastly, to avoid frequency-dependent properties of Bay K 8644,[14] we applied the two-pulse protocol at 0.1 or 0.2 Hz. The conditioning voltage interval of each two-pulse protocol was short enough, however, that Bay K 8644 might affect the amplitude of the inward current transient. The effect would be to diminish but not block the agonist effect of Bay K 8644 (i.e., "relative block", see Sanguinetti et al[14]) on the inward current transient and slow the time course of current recovery. A similar effect also could occur with EADs. Whether "relative block" develops by the second pulse of a two-pulse protocol

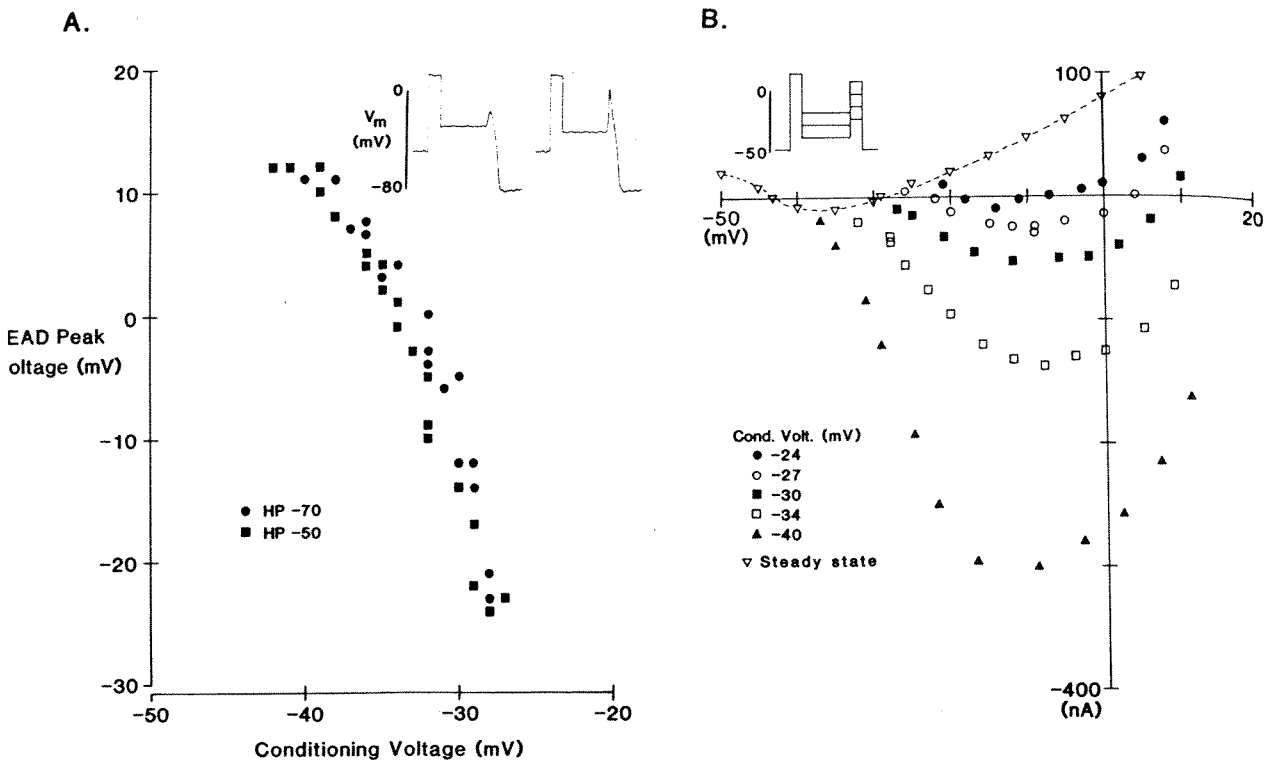


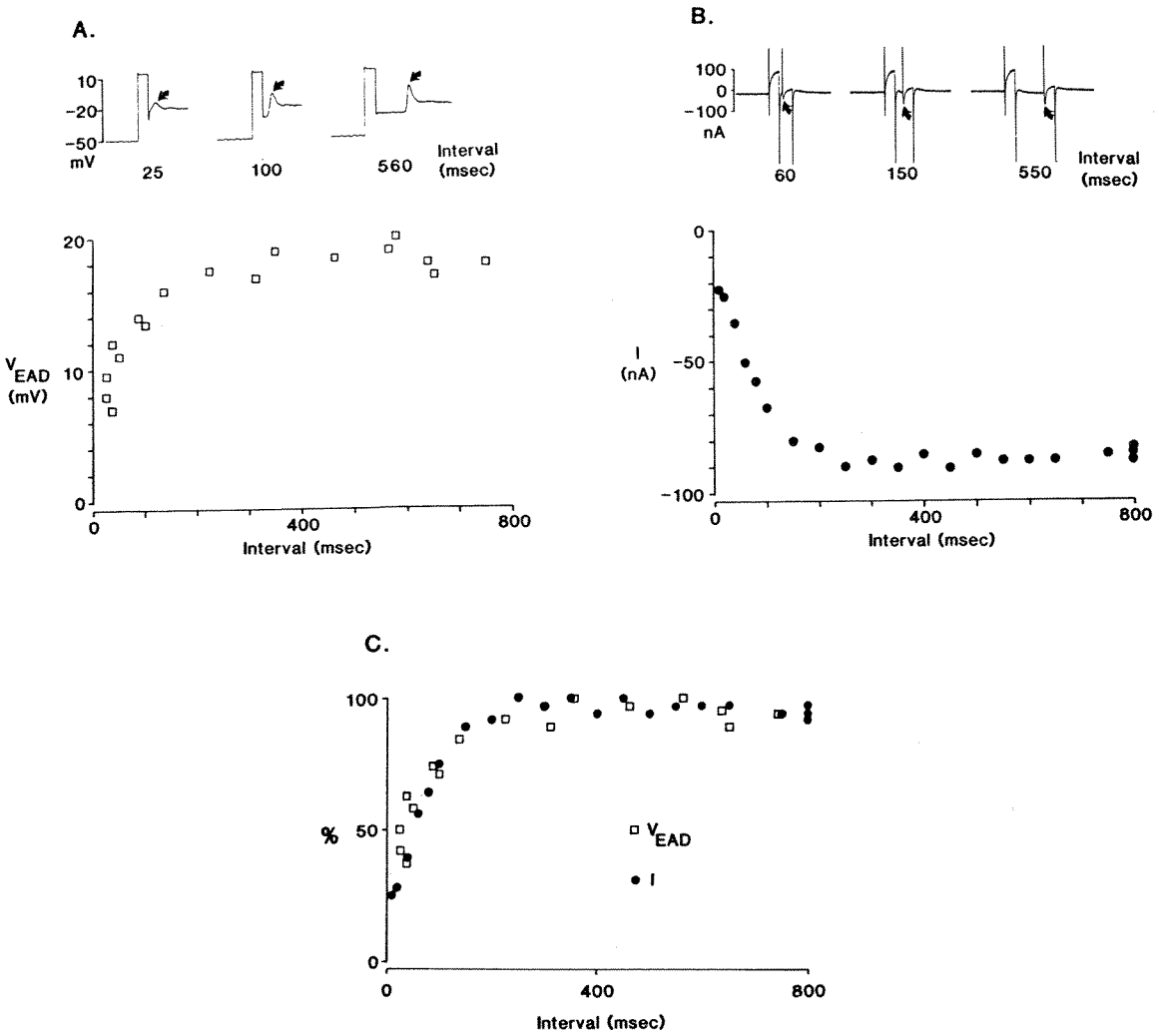

FIGURE 6. *Voltage-dependence of early afterdepolarizations (EADs) and the inward current transient in the same fiber. A: EADs were induced using the release from voltage-clamp protocol as shown in the inset (initial depolarizing step to +12 mV for 200 msec, conditioning steps for 600 msec to −28 and −33 mV, respectively). The EAD peak voltage was plotted against the conditioning voltage, and a steep inverse relationship was found. Similar results were obtained from holding potentials (HP) of −70 or −50 mV. B: Current-voltage relations for the peak inward current transient are shown. Each current-voltage relation was obtained by varying the amplitude of the test step of the two-pulse voltage clamp protocol, and several conditioning voltages were studied (see inset, conditioning step duration 600 msec). The size of the current-voltage relations was steeply dependent on the conditioning voltage. The steady-state current-voltage relation also is shown. See text for additional discussion.*

s not known, and this does not change our conclusion that an inward current displaying time- and voltage-dependent recovery properties underlies the EADs we studied. Relative block should dissipate during the several second pause before application of the next two-pulse protocol.

### *The Inward Current Transient is Carried Through $Ca^{2+}$ Channels*

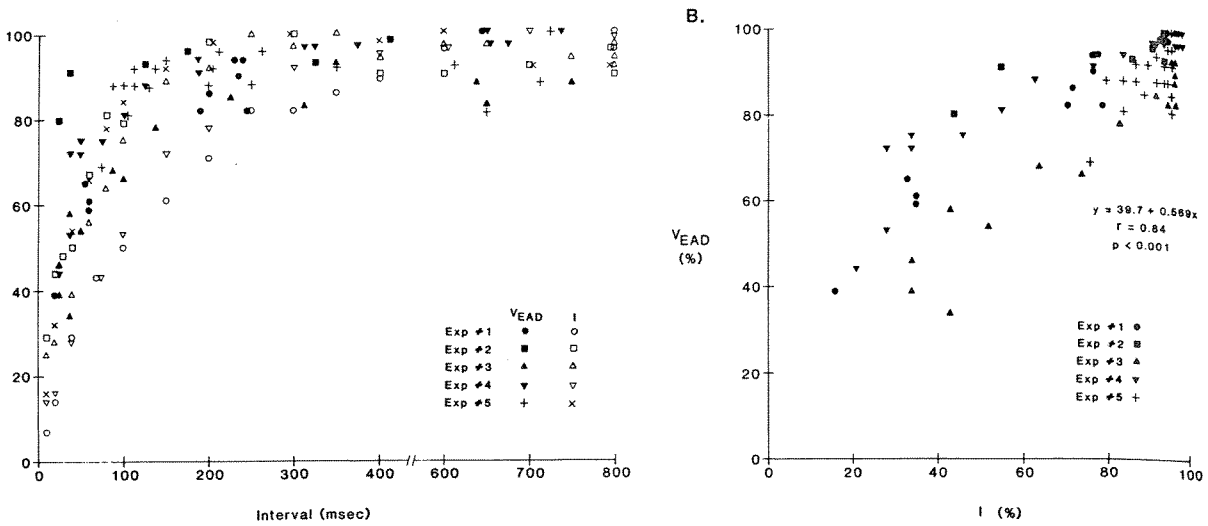
*Effect of TTX and nitrendipine on the inward current transient.* The effect of $Na^+$ channel block with TTX ($1\times10^{-5}$ M) and $Ca^{2+}$ channel block with nitrendipine ($2.5\times10^{-6}$ M) on the inward current transient was studied. Experimental results obtained in one fiber with use of the two-pulse voltage-clamp protocol are shown in Figure 9. The fiber was superfused with Bay K 8644-Tyrode's solution continuously. Panel A shows the two-pulse voltage clamp protocol and three current traces obtained with it. From a holding potential of −50 mV, the initial depolarizing step elicited an inward $Ca^{2+}$ current. Following a 600-msec long conditioning step to −37 mV, the test step elicited the inward current transient (arrow in top current trace). After 5 minutes' exposure to TTX, the inward current transient was not abolished (arrow, second current trace). TTX was then washed from the chamber and nitrendipine was added. Nitrendipine abolished completely the inward current transient (arrow, third current trace) as well as the inward surge of $Ca^{2+}$ current elicited with the initial depolarizing step. Current-voltage plots of the peak inward current transient obtained by varying the amplitude of the test step are shown in panel B. The current-voltage plot of the peak inward current transient obtained with Bay K 8644 was virtually unchanged by $Na^+$ channel block with TTX. In contrast, the inward current transient was abolished by $Ca^{2+}$ channel block with nitrendipine, leaving only outward current. Similar results were found in three fibers.

*Effect of $Na^+$-free Tyrode's solution on the inward current transient.* We studied whether the electrogenic Na-Ca exchange mechanism could carry the charge for the inward current transient. Conditions were established where the Na-Ca exchange mechanism should be inhibited by replacing all extracellular $Na^+$ with an impermeant cation (see Cannell and Lederer[22]). Experimental records are shown in




RE 7. *Time-dependence of recovery of early afterdepolarizations (EADs) and the inward current transient in the fiber in Bay K 8644-Tyrode's solution.* A: *EADs (arrows) were elicited with the release from voltage clamp protocol ferent conditioning step intervals (conditioning voltage −30 mV). Example records for intervals of 25, 100, and 560 are shown above and the amplitudes ($V_{EAD}$) were plotted against the conditioning voltage interval below. The itude of the EADs increased with lengthening of the conditioning step interval.* B: *The inward current transient ws) was studied by varying the conditioning voltage step interval of the two-pulse voltage-clamp protocol. Example ds for intervals of 60, 150, and 550 msec are shown above and the peak inward transient current (I) values were d against the conditioning voltage interval below. The amplitude of the inward current transient increased as the tioning step interval lengthened (same conditioning voltage as A).* C: *The time-dependent recovery of the EADS and ward current transient were normalized (see text) and replotted together against the conditioning voltage interval. ime courses of recovery were nearly identical.*

e 10A with Bay K 8644 continuously present. ıe top are shown typical current and tension ds obtained with the two-pulse voltage clamp ocol shown to the left. The inward current ient was elicited with the test step (arrow, first l). In addition, small amplitude twitches were nt with both depolarizing steps. Replacement $a^+$ with $Na^+$-free Bay K 8644-Tyrode's solu- to disable Na-Ca exchange, resulted in $Ca^{2+}$ ng of cells and an increase in twitch tension itude of nearly 20-fold. The inward current ient, however, was not abolished (large arrow, ıd panel). Consistent with cellular $Ca^{2+}$ overload was the development of $i_{TI}$ and an aftercontraction (small arrows, second panel). The return to $Na^+$-containing Bay K 8644-Tyrode's solution during washout resulted in return to the initial conditions with the inward current transient unchanged (arrow, third panel). Current-voltage plots for the peak inward current transient obtained by varying the voltage of the test step are shown below. The peak inward current transient was virtually unchanged in $Na^+$-free solution, conditions where the Na-Ca exchange mechanism and as well as $Na^+$ channel current should be absent. Similar results were obtained in four experiments.




3 8. *Normalized results for the time course of recovery of early afterdepolarizations (EADs) and the inward t transient in five fibers.* A: *Experimental results for EAD amplitude ($V_{EAD}$) and the amplitude of the inward current nt (I) were normalized for each experiment as was done previously, and show similar time courses of recovery.* B: *rcent recovery of EAD amplitude was plotted against the percent recovery of the peak inward current transient for ne conditioning step intervals. A highly significant correlation was present. See text for additional discussion.*

ıe depolarizing charge for EADs is carried ;h $Ca^{2+}$ channels, it should be possible to e EADs in $Na^+$-free solution. In $Na^+$-ning Bay K 8644-Tyrode's solution, an action ial triggered an EAD (Figure 10B, top panel). se from the voltage clamp protocol induced r EADs (middle panel, conditioning voltage ıV). In $Na^+$-free Bay K 8644-Tyrode's solu-ıe resting potential was depolarized yet release he voltage-clamp protocol again induced an The reduced EAD amplitude may have been l by the increased transient outward current a$^+$-free current record in Figure 10A; see also ll and Lederer[22]).

## Discussion

most important finding of these experiments is ntification of an inward current that underlies polarization of EADs: 1) The time-dependence overy from inactivation of the inward current nt and the time-dependence of recovery of EAD amplitude were nearly identical, and a highly significant correlation was present. 2) The amplitude of EADs and the inward current transient were steeply voltage-dependent over a similar range of conditioning voltages. 3) The voltage thresholds for the development of a net inward current transient and EADs were similar. 4) Without recovery of the inward current transient, EADs could not be elicited. Although latency and additional membrane currents may cause uncertainty about the precise quantitative relationship, we conclude that the depolarization of these EADs depended on the charge carried by the inward current transient.

Our results suggest that the inward current transient is a $Ca^{2+}$ current and that it is carried through L-type channels. The amplitude of the inward current transient was enhanced with Bay K 8644, and it was blocked by nitrendipine. Furthermore, the voltage range over which the inward current transient was elicited, as well as its sensitivity to dihydropyridines, is consistent with L-type $Ca^{2+}$ current.[14,23–26] The time course of the current, including its recovery from inactivation, is similar to that reported in isolated ventricular myocytes.[27] Although T-type $Ca^{2+}$ currents are large in cardiac Purkinje cells,[26] they activate at more negative voltages and are relatively insensitive to dihydropyridines. Bay K 8644 also affects $Na^+$ channel current,[28] however, the inward current transient is unlikely to be carried through $Na^+$ channels since it was not blocked by TTX in concentrations adequate to nearly completely abolish $Na^+$ current[29] or by the replacement of extracellular $Na^+$ by tetramethylammonium. In addition, $Na^+$ current should remain nearly completely inactivated at voltages where recovery of the inward current transient occurred.[30] The removal

. **Correlation of Time-Dependence of Recovery Between fterdepolarization Amplitude and the Peak Inward Cur-ınsient**

| covery | Regression equation | Correlation coefficient | *p* value |
|---|---|---|---|
| release | $y=39.7+0.57X$ | $r=0.84$ | <0.001 |
| dV/dt max | $y=-30.3+1.21X$ | $r=0.75$ | <0.001 |
| EAD peak | $y=12.9+0.81X$ | $r=0.65$ | <0.001 |

r regression analysis results for correlation between terdepolarization (EAD) recovery and the inward current t. The recovery interval for the inward current transient duration of the conditioning voltage step. The recovery for EADs was measured three ways as described in the

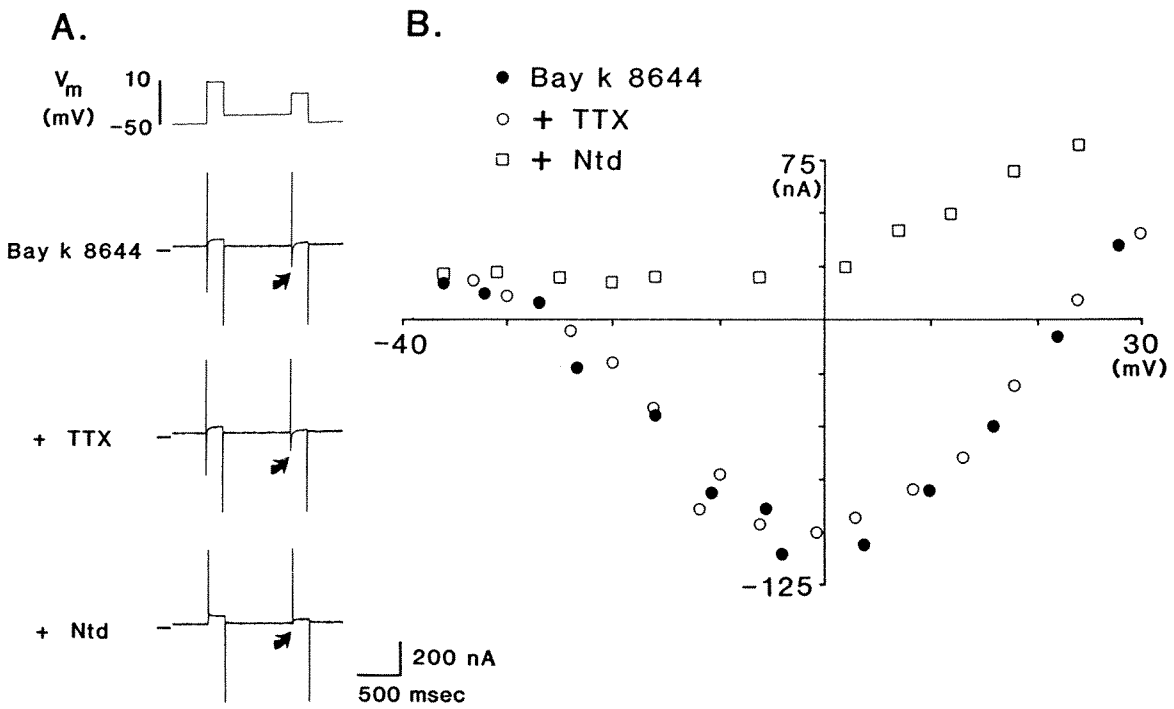



FIGURE 9. *Block of the inward current transient by nitrendipine (Ntd) but not by tetrodotoxin (TTX).* A: *Membrane current records were obtained using the two-pulse voltage-clamp protocol shown (initial depolarizing step to +10 mV for 100 msec, conditioning step to −37 mV for 600 msec, continuous Bay K 8644-Tyrode's superfusion). Following the conditioning step, the test step to −15 mV elicited a large amplitude inward current transient (arrows). The addition of TTX ($1\times10^{-5}$ M) failed to block the inward current transient. After washout of TTX, nitrendipine ($2.5\times10^{-6}$ M) abolished the inward current transient. Nitrendipine also blocked the $Ca^{2+}$ current elicited with the initial depolarizing step from the holding potential of −50 mV.* B: *Peak current-voltage plots for the inward current transient were obtained in the same fiber by varying the amplitude of the test step. TTX had little effect on the inward current transient, whereas, it was abolished by nitrendipine.*

of extracellular $Na^+$ also should disable the electrogenic Na-Ca exchange mechanism. While this resulted in a marked increase in twitch tension amplitude consistent with $Ca^{2+}$ loading of cells, inhibition of Na-Ca exchange failed to abolish the inward current transient.

Our results in fibers with EADs (see Figure 3B) show the absence of an oscillatory current similar to $i_{TI}$, the current that underlies delayed afterdepolarizations (for review, see Wit and Rosen[31] and January and Fozzard[32]). Furthermore, $i_{TI}$ and its after-contraction could be induced independently in fibers that already had EADs (see Figure 10). We obtained similar findings using acetylstrophanthidin ($1.5\times10^{-7}$ M, see Terek and January[33]) to inhibit the Na-K pump (data not shown). These findings support the conclusion that $Ca^{2+}$ overload with the concomitant induction of an $i_{TI}$-like current is not required to elicit the EADs we studied.[3] A similar conclusion was reached by Marban and coworkers[6] for EADs induced in ventricular muscle with $Cs^+$.

Bay K 8644 produced an inward shift in the steady-state current-voltage relation near action potential plateau voltages, which can account for lengthening of action potential duration. Since Bay K 8644 produces long openings of L-type $Ca^{2+}$ channels and these channels may reopen,[23,24] the macroscopic current changes we measured may result from the increased channel mean open times. Bay K 8644 also has been shown to shift the steady-state activation and inactivation relationships[14,15] which could modify the number of $Ca^{2+}$ channels available to open. Our results, however, do not distinguish these possibilities from other possibilities including altered probability of channel (re)openings or closings, changes in single-channel conductance, and alterations in other membrane currents. Drug and ionic interventions that suppress EADs and shorten action potential duration (TTX, lidocaine, nitrendipine, verapamil, and raising $[K]_o$; see January et al[3]), were all shown to reversibly shift the steady-state current-voltage relation outwardly near action potential plateau voltages. Although interpretation of these observations is complex, they are consistent with known roles for several membrane currents in the regulation of the action potential plateau including $Na^+$ "window" or slowly inactivating current,[34,35] $Ca^{2+}$ current,[15,36,37] and $K^+$ currents.[38–42] At low depolarization frequencies, or following pauses, transient outward current ($i_{TO}$)[13] also is likely to contribute increasingly to the shape of the plateau. Additionally, electrogenic currents from the Na-K pump[43]

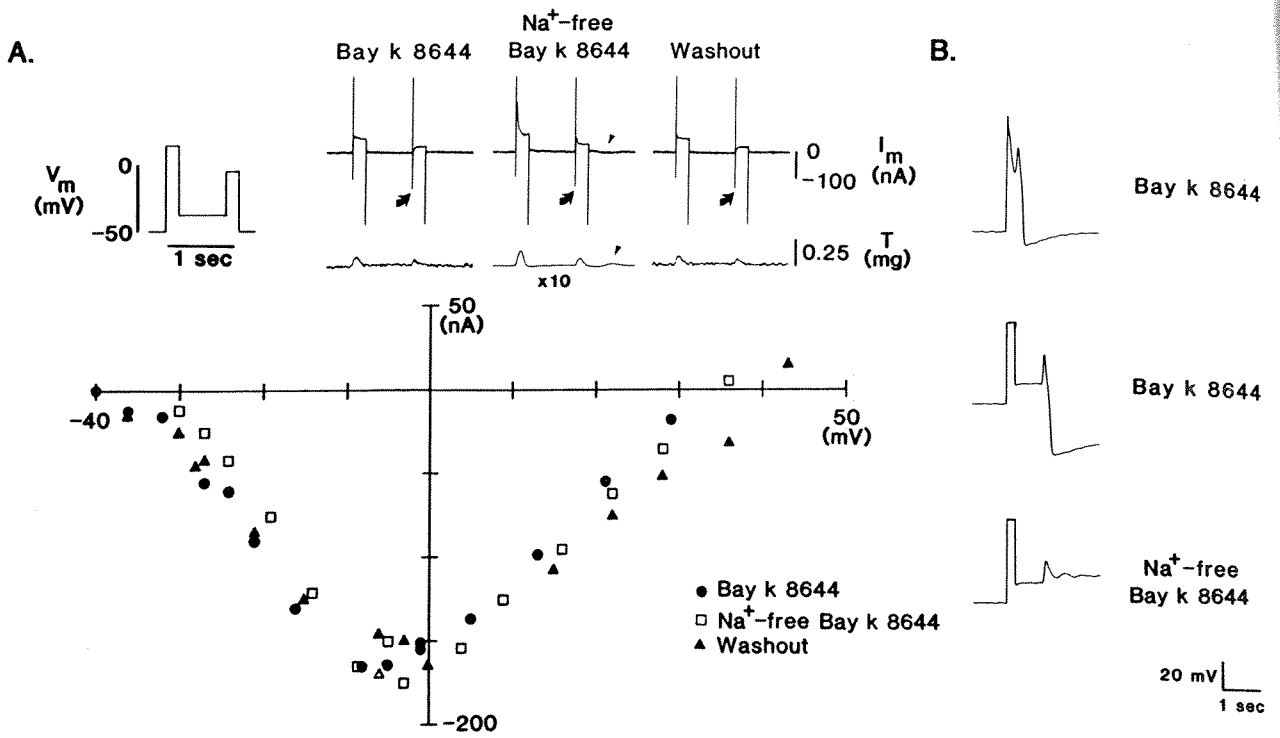



FIGURE 10. *The effect of $Na^+$-free solution.* A: *The two-pulse voltage-clamp protocol (see inset) was used to obtain membrane current ($I_m$) and twitch tension (T) records. From the holding potential of −50 mV, the initial depolarizing step to +12 mV for 200 msec was followed by a conditioning step to −38 mV for 600 msec. The test step to −10 mV elicited a large amplitude inward current transient in Bay K 8644-Tyrode's solution (arrow, first panel). After $Na^+$ was removed from the bath (second panel), $Ca^{2+}$ overload caused twitch tension to increase nearly 20-fold (note the 10-fold change in the calibration). The inward current transient was virtually unchanged (large arrow). An aftercontraction and $i_{Ti}$ also developed (small arrows), consistent with $Ca^{2+}$ overload. Washout to Bay K 8644-Tyrode's solution (third panel) reversed the effects of $Na^+$ withdrawal. The current-voltage plots below show that $Na^+$ replacement with $Na^+$-free Bay K 8644-Tyrode's solution had little effect on the peak inward current transient.* B: *EADs were elicited with action potentials (upper trace) or following release from voltage clamp (middle trace) in Bay K 8644-Tyrode's solution. In $Na^+$-free Bay K 8644-Tyrode's solution (lower trace), EADs could still be elicited using the release from voltage clamp protocol. See text for additional discussion.*

and the Na-Ca exchange mechanism,[44] as well as intercellular $K^+$ accumulation during depolarization,[45] also are likely to contribute. The possibility that some of the interventions we studied could interact directly with the Bay K 8644 effects on $Ca^{2+}$ channels can not be excluded.

### *Can L-Type $Ca^{2+}$ Channels Provide Depolarizing Current for EADs? Role of Recovery From Inactivation and "Window" Current*

Our results suggest that recovery of L-type $Ca^{2+}$ channels from inactivation is an essential step in EAD development. L-type $Ca^{2+}$ current was activated with the initial large depolarizing step of the two-pulse protocol, and at physiological temperatures and with $Ca^{2+}$ as the charge carrier it then rapidly decayed (see Figures 1 and 9A) as $Ca^{2+}$ channels inactivated (see Kass and Sanguinetti[46] and Lee et al[47]). The conditioning step then allowed for their recovery from inactivation.

In the two-pulse voltage-clamp protocol, small amplitude depolarizing test steps were used to voltage activate the inward current transient. In a non–voltage-clamped preparation, however, the mechanism of the transition from action potential repolarization to EAD depolarization is less clear. One possible mechanism is that with the time- and voltage-dependent recovery of L-type $Ca^{2+}$ channels from inactivated to closed states, an inward current could gradually develop if these channels also can reopen. This requires overlap of the activation and inactivation relationships, thereby permitting channel reopening(s) producing a steady-state or "window" current. Thus, repolarization into the "window" could lead to recovery of an inward current. The presence of a steady-state component of inward $Ca^{2+}$ current is supported by measurements of activation and inactivation in several types of cardiac tissue[15,42,48,49] and by single channel recordings.[50,51] Furthermore, maintained inward $Ca^{2+}$ current within this "window" has been shown directly for normal and dihydropyridine treated $Ca^{2+}$ channel currents.[15] Once EAD depolarization is initiated voltage-dependent activation of L-type $Ca^{2+}$ current also could occur.

### *Interaction With the Steady-State Current*

We have postulated previously that the induction of EADs may be considered as the interaction of two

phases, an initiation or conditioning phase that leads into an EAD depolarization phase.[3] The conditioning phase is composed of those ion channel and electrogenic pump currents (each having time- and voltage-dependence) whose balance controls the shape of the action potential plateau. The EAD depolarization phase is dependent on the availability of L-type $Ca^{2+}$ current to carry depolarizing charge.

Because several membrane currents may participate in EADs, we performed most experiments in Tyrode's solution to retain the normal currents present at action potential plateau voltages. The most positive voltage where we obtained EADs was close to the voltage at which recovery of the inward current transient was adequate to provide a net inward current. In contrast, the most negative voltage from which EADs could be elicited was that voltage where the negative slope region of the steady-state current-voltage relation crossed the zero current axis. Below this voltage, repolarization was unavoidable despite increased recovery from inactivation of the inward current transient. Comparison of the voltage-dependence of EAD amplitude with the voltage-dependence of the inward current transient also is of interest. Although large amplitude inward current transients could be generated with depolarizing voltage steps from more negative conditioning voltages, recovery from inactivation of only small amplitude inward current transients was required to induce EADs. This suggests that recovery from inactivation and reactivation of only a relatively small fraction of $Ca^{2+}$ current is needed to shift the balance of membrane currents during the action potential plateau towards depolarization.

These results provide a framework for interpreting the roles of action potential duration and plateau voltage, and the underlying membrane currents, in the induction and block of these EADs. Since the time-dependence of recovery from inactivation of L-type $Ca^{2+}$ current is slow near action potential plateau voltages (Figures 7 and 8, see also Isenberg and Klockner[27]), interventions that retard action potential repolarization in the voltage range from which EADs initiate should augment channel recovery and the development of EADs. This mechanism could account for the induction of EADs by antiarrhythmic drugs that prolong action potential duration near plateau voltages. In contrast, interventions that shift the steady-state current-voltage relation outwardly result in more rapid repolarization through the voltage range from which EADs can occur. Less time for channel recovery is available which should suppress EADs (indirect block of EADs). Interventions also may directly suppress L-type $Ca^{2+}$ channel current (direct block of EADs). This hypothesis is consistent with our previous observations,[3] and it may underlie EADs arising at plateau voltages with other EAD models.

Our results suggest that during action potential repolarization, recovery from inactivation of L-type $Ca^{2+}$ current within its "window" may supply depolarizing charge for the EADs we studied. Although L-type $Ca^{2+}$ channels are known to participate in the maintenance of the action potential plateau, our results suggest a new role for $Ca^{2+}$ channels, one in which recovery from inactivation during repolarization can lead to recovery of additional inward current. This suggests an important role for $Ca^{2+}$ channel current in the generation of certain types of triggered automaticity. Furthermore, this mechanism of recovery from inactivation of inward current within its "window" also could contribute to the shape and duration of the normal cardiac action potential. Clearly, the mechanism suggested by our results requires additional study.

## Acknowledgments

We thank Dr. J.C. Makielski for helpful discussion during the course of these experiments and for reading of the preliminary manuscript. We also thank Dr. A. Scriabine of Miles Laboratories, Inc, for the gift of Bay K 8644.

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KEY WORDS • Purkinje fibers • early afterdepolarizations • membrane currents • calcium currents • Bay K 8644 • arrhythmia