brief communication

Ca channel gating during cardiac action potentials

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ABSTRACT How do Ca channels conduct Ca ions during the cardiac action potential? We attempt to answer this question by applying a two-microelectrode technique, previously used for Na and K currents, in which we record the patch current and the action potential at the same time (Mazzanti, M., and L. J. DeFelice. 1987. *Biophys. J.* 12:95–100, and 1988. *Biophys. J.* 54:1139–1148; Wellis, D., L. J. DeFelice, and M. Mazzanti. 1990. *Biophys. J.* 57:41–48). In this paper, we also compare the action currents obtained by the technique with the step-protocol currents obtained during standard voltage-clamp experiments. Individual Ca channels were measured in 10 mM Ca/1 Ba and 10 mM Ba. To describe part of our results, we use the nomenclature introduced by Hess, P., J. B. Lansman, and R. W. Tsien (1984. *Nature (Lond.)*. 311:538–544). With Ba as the charge carrier, Ca channel kinetics convert rapidly from long to short open times as the patch voltage changes from 20 to −20 mV. This voltage-dependent conversion occurs during action potentials and in step-protocol experiments. With Ca as the charge carrier, the currents are brief at all voltages, and it is difficult to define either the number of channels in the patch or the conductance of the individual channels. Occasionally, however, Ca-conducting channels spontaneously convert to long-open-time kinetics (in Hess et al., 1984, notation, mode 2). When this happens, which is about once in every 100 beats, there usually appears to be only one channel in the patch. In this rare configuration, the channel is open long enough to measure its conductance in 10 Ca/1 Ba. The value is 8–10 pS, which is about half the conductance in Ba. Because the long openings occur so infrequently with Ca as the charge carrier, they contribute negligibly to the average Ca current at any particular time during an action potential. However, the total number of Ca ions entering during these long openings may be significant when compared to the number entering by the more usual kinetics.

INTRODUCTION

Ba currents through L-type Ca channels demonstrate three kinds of gating kinetics in the heart: mode 1 gating with brief, low-probability openings; mode 2 gating with long-lasting openings interrupted by brief closings; and the null mode with no openings (Hess et al., 1984). According to this classification scheme, dihydropyridine agonists, such as Bay K 8644, enhance the Ba current by favoring mode 2. Antagonists, like nitrendipine, inhibit current by favoring the null mode. Long-lasting openings occasionally occur in the absence of Bay K (Hess et al., 1984), suggesting that switching between modes may be regulated by intrinsic mechanisms (Nowycky et al., 1985).

Although this classification scheme is in dispute (Brown et al., 1984; Cavalie et al., 1986; Lacerda and Brown, 1989), many authors support it (Tsien et al., 1985; Reuter et al., 1986; Kokubun et al., 1986; Yue et al., 1989). For example, Yue et al. demonstrate that β-adrenergic stimulation of Ca channels occurs by discrete jumps to higher modes rather than by graded increases in channel kinetics. Because stimulation of an endogenous pathway promotes the long openings, Yue et al. (1989) speculate that mode 2 gating may have a physiological role in the heart (see also Hess et al., 1986*b*; Tsien et al., 1986). Brum et al. (1984) conclude that β-adrenergic stimulation increases the probability that the Ca channel is in the open state. To separate Ca currents from other types of current, it would be advantageous to study single-channel events. However, it is difficult to measure individual Ca channels without using high concentrations of Ba or Ca to increase the conductance, and without using an agonist to increase the open times. Using Ba or other surrogate ions is undesirable because these substitutions alter channel kinetics, and dihydropyridines leave open the question of the native state of the channel. Previous experiments in low concentrations of Ca or Ba rely on (+)-PN 202-791 or Bay K 8644 to resolve single-channel events (Friel and Tsien, 1989; Yue and Marban, 1990).

In this paper we study single-channel events in 10 Ba and 10 Ca/1 Ba in the absence of dihydropyridines. We provide no direct evidence for or against models that switch between discrete kinetic schemes (e.g., the modes of Yue et al., 1989) versus models with graded kinetics within a single scheme (e.g., Lacerda and Brown, 1989). The characteristics of Ca channels we describe can generally be explained in conventional terms of voltage-dependent activation. Inactivation is more prominent with Ca as the charge carrier, as might be expected for

Ca-dependent inactivation. Perhaps the only feature of Ca channels that may fall under the term "modes" is the occasional failure of the channels to inactivate when Ca is the charge carrier. Patlak and Ortiz (1986) describe a similar phenomenon for Na channels. L-Type Ca channel gating in Ba appears to be homogeneous, with smooth but steeply voltage-dependent transitions from brief to long open times. Gating in Ca is qualitatively different. At any potential the channel generally exhibits brief bursts but in 1% of the sweeps will exhibit long-lasting openings. Regardless of the specific kinetic interpretation, dihydropyridines and β-adrenergic stimulation promoted long openings. We have asked whether these long openings occur when the Ca channel carries CA under physiological conditions. Is so, what controls them in the absence of drugs, and what is their significance for cardiac excitability?

To answer these questions we compared Ba and Ca currents in beating and nonbeating cells. Contrary to our expectations, when Ca channels carry Ba with no exogenous agonists present, the channels are always open during the relatively extended plateau phase of action potential. They switch from this long-lasting opening to brief openings and closings during the late repolarization phase. This conversion occurs over a narrow range of voltage between 20 and -20 mV in both beating and nonbeating cells. When the Ca channel carries Ca it is rarely open for long times. Prolonged openings do occur sporadically, and but for smaller amplitude they are approximately like the Ba openings. We conclude that Ba is the charge carrier; positive membrane potential (as occurs during an action potential) evokes long open-time kinetics with high probability. On the other hand, when Ca is the charge carrier the probability that a long opening occurs is quite low. Because these long openings are sporadic and qualitatively different than the usual Ca kinetics, we refer them, in likeness to Hess et al. (1984), as mode 2 openings. Under this interpretation mode 2 gating is intrinsic to the Ca channel and may play a role in transporting Ca ions into the cell, even in the absence of β-adrenergic stimulation.

METHODS

Embryonic ventricle cells were prepared by enzymatic digestion of 7-d-old chick embryo hearts, following the procedure of Fujii et al. (1988). After 12–24 h in tissue culture medium, and immediately preceding the experiments, we washed the cells with bath solution and performed the experiments at room temperature. The composition of the bath solution in millimolar units was: 130 Na, 1.3 K, 1.5 Ca, 0.5 Mg, 5 dextrose, and 10 Hepes, adjusted to 7.35 pH and 273 mOsm; all anions are Cl except 0.5 SO_4 and 1.3 PO_4. The whole-cell electrode for measuring voltage contained an intracellularlike solution consisting of 120 K, 0.1 Ca, 2 Mg, 1.1 EGTA, and 10 Hepes, adjusted to 7.35 pH and 268 mOsm; all anions are Cl. The cell-attached electrode for measuring patch currents contained one of two solutions: 10 Ba, 110 Na (called 10 Ba); or 10 Ca, 1 Ba, 108 Na (called 10 Ca); each solution also contained 0.5 Mg, 1.3 K, 0.01 TTX, 5 TEA, 10 4-AP, 10 Hepes, and 5 dextrose, adjusted to 7.35 pH and 273 mOsm. During action potentials, the inward Na current subsides in <4 ms after the upstroke of the action potential (Wellis et al., 1990). The outward Na current lasts up to 40 ms, but it is <0.4 pA in 130 mM Na and 0 TTX. In the present experiments we expect negligible contamination from fast Na currents. An outward current flows through inward–rectifier channels during the plateau phase of the action potential (Mazzanti and DeFelice, 1990). Although this current is expected to be small in 1.3 mM K, we included 1 mM Ba in the 10 Ca pipette to eliminate any possibility of contamination.

In these experiments we use two electrodes to record the patch current and the whole-cell voltage. The data were bandlimited at 1,000 Hz, and we have subtracted the capacitive transient from all current traces. For a detailed description of the two-electrode recording method during action potentials, see Mazzanti and DeFelice (1987, 1988) and Wellis et al. (1990). A plot of individual patch current, $i(t)$, against the cell action potential, $V(t)$, gives the instantaneous (or open-channel $i(V)$ curve. After averaging the patch current over N action potentials, plotting the mean current, $I(t)$, against $V(t)$ results in the average $I(V)$ curve during the action potential. If there were one channel in the patch, $I(V)$ would reflect the behavior of N channels in the unpatched membrane. In the present experiments the patch pipette is always clamped to the bath potential. $I(V)$ and $i(V)$ are dynamic currents being swept out during the action potential. In 7-d-old chick ventricle, which is a transitional stage in development, the cells beat (and often stop beating) spontaneously. We take advantage of this behavior to work on both types of cells, comparing the currents during action potentials with the currents from the same patch during step-voltage protocols.

The electrodes were made from borosilicate glass (model 7052, Corning Glass Works, Corning, NY) using a programmable puller (Sachs-Flaming, PC-84, Sutter Instruments). After coating with Sylgard (Dow Corning Corp. Midland, MI) and fire polishing the tip to ~1–2 μm internal diameter, the electrodes had resistances of 4–10 megaohms. The cells are ~10–13 μm in diameter, and the tips of the current and voltage electrodes could be placed 5–10 μm apart. List EPC5 and EPC7 amplifiers were used to measure the voltage and current, respectively. We stored the data on a VCR, and analyzed it on an oscilloscope, (model 4049; Nicolet Scientific Corp. Northvale, NJ) and an IBM-AT (IBM Instruments Inc., IBM Corp. Danbury, CT), using programs developed in house.

RESULTS

Fig. 1 *a* illustrates an action potential from a single cardiac cell beating spontaneously in normal bath solution at room temperature. The trace below the action potential is the current through a cell-attached patch during this particular beat. The voltage is clamped to virtual ground, thus, the potential across the patch membrane is the action potential. The sharp downward spike in the patch action current that occurs at the foot of the cell action potential indicates that the channel is open just before the upstroke. Inward Na current, which is diminished in these experiments by TTX, would in any case decline to zero within 4 ms after the upstroke (Wellis

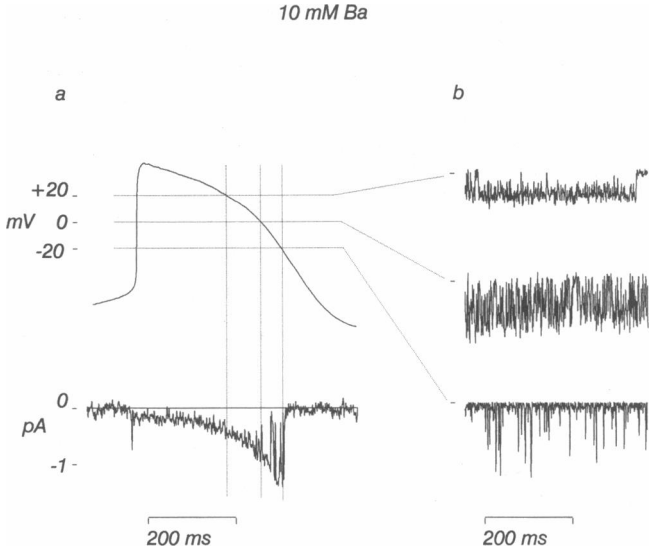

FIGURE 1 (*a*) Whole-cell action potential and 10 mM Ba action current from a patch on a spontaneously beating 7-d-old chick ventricle at room temperature. (*b*) Currents from the same patch elicited during voltage steps applied to the patch after the cell stopped beating. The holding potential was −80 mV (near the cell resting potential), and the three test potentials were 20, 0, and −20 mV. The lines connect corresponding voltages.

et al., 1990; see Methods). During the rapid depolarization phase, the current is first reduced with respect to the initial downward spike. During the remainder of the action potential, the current gradually increases. These changes in the current amplitude reflect the changing driving force in the patch. In this particular case, the channel is open during virtually the entire action potential; note that the current begins to flicker toward the end of the repolarization phase, just before the channel closes. This pattern is representative of all Ba action currents, though the time at which the channel closes varies from beat to beat. We think only one channel was in the patch because we never saw multiple conductance levels. These long openings, which typically last ~300 ms, are always interrupted by brief closings that are more frequent during late repolarization.

Within seconds after the cell stopped beating, we voltage clamped the same patch (Fig. 1 *b*). Stepping from −80 to 20 mV produced long openings like the one illustrated in the top trace in *b*. At the beginning of this step the channel is open; it closes briefly and then remains essentially open nearly to the end of the test pulse. This long opening is interrupted by several brief closings that are limited by the bandwidth of the recording (1,000 Hz). This same pattern appears in virtually every step to positive potentials. Stepping the potential to 0 mV generates a larger, rapidly flickering current (same bandwidth); stepping it −20 mV evokes an even larger current whose brief openings are too short to resolve. The lines connect the currents during step experiments with the currents at analogous voltages during the action potential. There is a rough correspondence between the records in *b* and *a*: the long opening at 20 mV mirrors the current flowing during the plateau, and the brief openings at −20 mV reflect the flickering during late repolarization. The transition occurs within 300 ms. Thus, in addition to the change in the current amplitude, which is explained by the shift in driving force, there is a rapid change in the kinetics of the Ba current that would appear to be strictly voltage dependent.

An entirely different pattern emerges with 10 Ca/1 Ba in the patch pipette. The action currents are nearly always made up of low-amplitude, relatively brief openings that are confined to the plateau phase (Fig. 2 *a*). Unlike Ba currents, Ca currents decrease during repolarization though the driving force, for Ca increases. This apparent inactivation of a multichannel current is also evident in the step-voltage experiments. Whereas the Ba current is virtually constant throughout the 20-mV pulse (Fig. 1 *b*), the Ca current decays in ~100 ms (Fig. 2 *b*). Furthermore, Ba currents nearly always appear to be composed of one or two discrete levels, but Ca currents rarely resolve into fixed conductances.

A rare opening does occur in 10 Ca/1 Ba that is similar

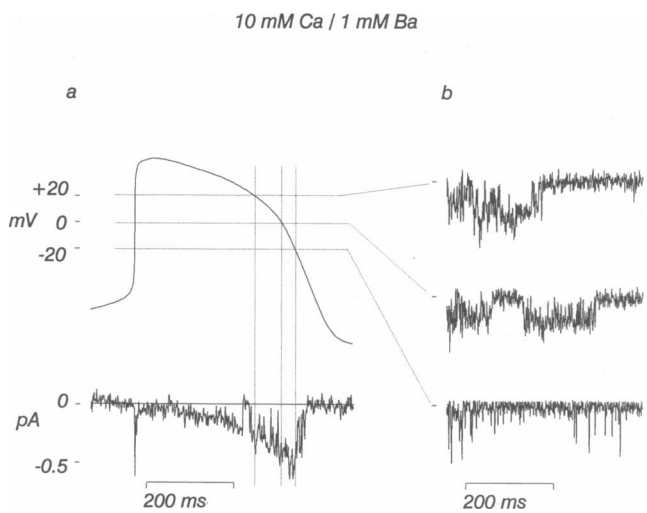

FIGURE 2 (*a*) Whole-cell action potential and 10 mM Ca/1 mM Ba action current from a patch on a spontaneously beating 7-d-old chick ventricle at room temperature. (*b*) Currents from the same patch elicited during voltage steps applied to the patch after the cell stopped beating. The holding potential was −80 mV (near the cell resting potential), and the three test potentials were 20, 0, and −20 mV. The lines connect corresponding voltages.

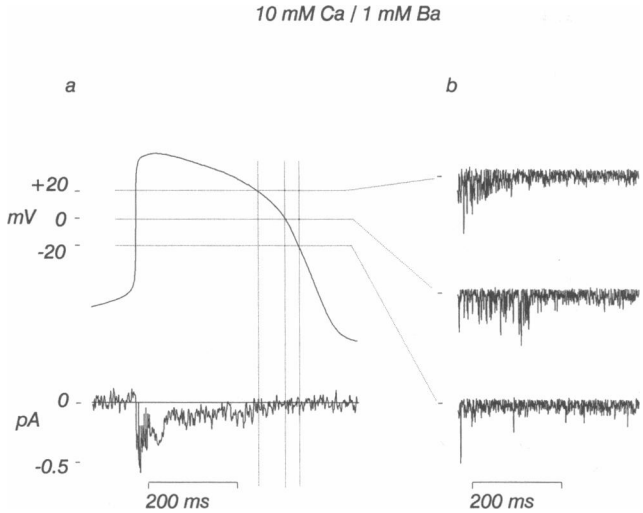

FIGURE 3 The same experiment as the one described in Fig. 2, but now showing selected rare openings for (*a*) the action current and (*b*) the step currents.

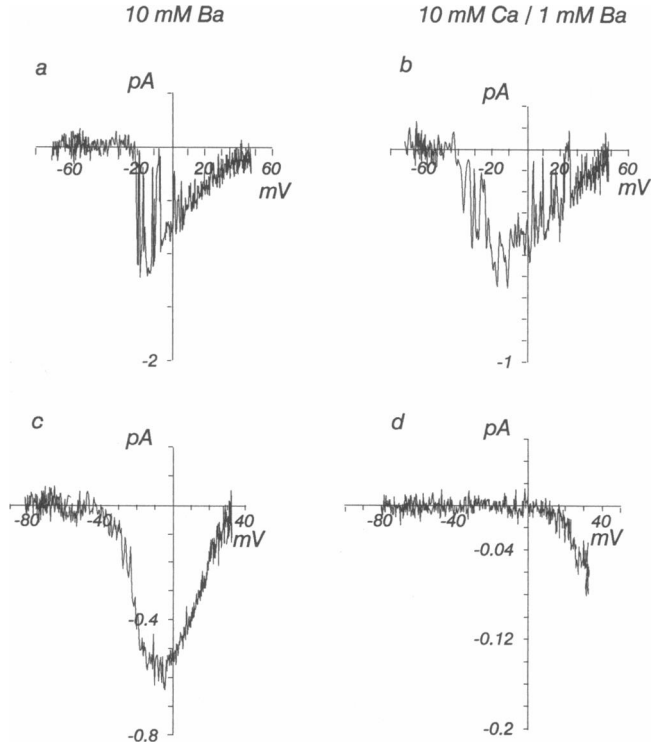

FIGURE 4 A summary of the experiments in the preceding figures. (*a*) and (*b*) are the instantaneous $i(V)$ curves, which are defined as the action current versus the action potential for a particular beat. In 10 Ba (*a*) the $i(V)$ curve is representative of each beat; in 10 Ca/1 Ba (*b*) the $i(V)$ curve represents the rare openings that occur sporadically. (*c*) and (*d*) are the mean $I(V)$ curves, which are defined as the average action current over 20 beats versus the action potential; (*d*) includes in the average one of the rare mode 2 openings, giving it approximately five times its normal weight. Note that in 10 Ba the $I(V)$ curve peaks midway during repolarization; in 10 Ca/1 Ba the $I(V)$ curve is practically zero everywhere, except during the early plateau phase of the action potential. If the Ca action currents included more of the long openings, (*d*) would approach the shape of (*c*).

to the normal opening in 10 Ba. Fig. 3 is from the same patch as Fig. 2, but the traces were selected by their analogy to Ba currents. Notice that there now appears to be one major conductance state in the patch action current. Furthermore, the channel seems to be open throughout the plateau phase of the action potential, it begins to flicker during late repolarization, and then it closes. Except for amplitude, this pattern is virtually identical to the Ba current. The correspondence is less striking for the voltage-clamp experiments, but here too there is a proximate similarity. Thus, comparable kinetics exist for either divalent ion, however, the long openings that are common in Ba are scarce in Ca.

To summarize the results in Ba an Ca, we plot current-voltage relationships for the two cases. Fig. 4, *a* and *b*, compare the instantaneous $i(V)$ curve of the normal Ba current (Fig. 1) with the instantaneous $i(V)$ curve of the rare Ca current (Fig. 3). The Ba consuctance measured by drawing a line by eye through the center of the open-channel currents is 15–20 pS ($n = 50$). The Ca conductance is 8–10 pS ($n = 12$). Panels *c* and *d* compare the average $I(V)$ curves for the same two experiments for all openings. In 10 Ba, or in 10 Ca/1 Ba, the $i(V)$ curves are analogous, though the Ba current is twice as large. The Ba closing is generally more abrupt, and Ba currents rectify inwardly as they approach zero. The $I(V)$ curves, on the other hand, are totally different. The average Ba current peaks at about -10 mV, midway through the repolarizaiton phase, whereas the average Ca current is practically zero throughout the action potential, except during the early plateau phase. This pattern is virtually identical in 50 experiments in 10 Ba and 12 experiments in 10 Ca/1 Ba, with minor variations due to variability in action potential shape.

DISCUSSION

A wide variety of cardiac cells express both L-type and T-type Ca channels (Bean, 1985; Nilius et al., 1985; Mitra and Morad, 1987; Hagiwara et al., 1988; Hirano et al., 1988, 1989; Kawano and DeHaan, 1989). The relative proportion of these two kinds of channels generally depends on the class of heart tissue, but in most cells the predominant type is L. Kawano and DeHaan (1989) report that in chick ventricle at 14 d, the major channel is type T. Kristof et al. (1990) find that in the same tissue at 6-d, the principal channel is type L. We use isolated

7-d-old chick ventricle cells that have been in tissue culture 1 or 2 d. The channels we have studied usually inactivate in 10 Ca/1 Ba, but not in 10 Ba. They have a Ca conductance of 8–10 pS (measured during the rare times the Ca current lasts long enough), and they have a Ba conductance of 15–20 pS. These characteristics are typical of L-type Ca channels.

Dihydropyridines are useful in resolving channel currents because they promote long openings. In addition, their effect on Ca channels helps understand the mechanism of action of these important compounds. Hess et al. (1984) favor a model in which Bay K 8644 induces a discrete shift from one kinetic mode to another. Brown et al. (1984) and Lacerda and Brown (1989) advocate a single kinetic scheme in which dihydropyridines increase the probability that a closed channel reopens. The common observation is that when L-type channels are conducting Ba, dihydropyridines promote long openings. Interestingly, the long openings occur in the absence of Bay K 8644 (Hess et al., 1984) and in the presence of isoproterenol (Yue et al., 1989), suggesting they are indigenous to the channel and play a physiological role.

Two features of Ca channels relate to this suggestion: Ca-mediated inactivation, and Ca conductance versus Ba conductance. Brehm and Eckert (1978) and Brehm et al. (1980) first described Ca-mediated inactivation in *Paramecium*. Injecting EGTA or reducing the influx of Ca diminishes inactivation, and increasing Ca entry enhances inactivation. These experiments suggest that the cytoplasm incompletely buffers Ca ions entering the cell, thus allowing concentration domains to form near individual channels (Chad and Eckert, 1984). Ca-mediated inactivation also exists in heart cells (Kohlhardt et al., 1975; Shimoni, 1981; Josephson et al., 1984), where Ca current appears to be inhibited by the Ca ions near the internal membrane (Argibay et al., 1988). Thus, we interpret the long openings in Ba as the result of (*a*) voltage activation of the channel, and (*b*) the inability of Ba to inactivate the channel. The effect must be local, at least on the scale of the patch dimension, because the surrounding membrane contains channels conducting the ions present in normal saline, including Ca.

Ca channels are often studied in 20–110 mM Ba to generate more resolvable currents (Reuter et al., 1982; Tsien et al., 1983; Cavalie et al., 1983). Ba ions have the additional advantage of reducing, if not entirely eliminating, inward-rectifier channels common to heart tissue (Sakmann and Trube, 1984*a*, *b*; Kell and DeFelice 1988). In Ca, the conductance is lower than in the same concentration of Ba (Cavalie et al., 1983; Nilius et al., 1985; Hess et al., 1986*a*; Yue and Marban, 1990). For example, in guinea-pig ventricle the conductance of L channels is 9.7 pS in 110 Ca, but 29.1 pS in 110 Ba. Our 10 mM Ca solution also contains 1 mM Ba, and the question arises whether its presence influences the Ca current. In mixtures of Ca and Ba, Yue and Marban (1990) find that single-channel currents are never smaller than in pure Ca. These results are contrary to those of Hess and Tsien (1984), McDonald et al. (1986), and Campbell et al. (1988). Friel and Tsien (1989) attempt to resolve this discrepancy by investigating the anomalous mole fraction effect under conditions that distinguish channel permeation from channel gating. In PC-12 cells at 0 mV, their unitary currents were 0.25 pA (10 Ca/0 Ba), 0.21 pA (3 Ca/7 Ba), and 0.55 pA (0 Ca/10 Ba). Under similar conditions, Yue and Marban (1990) report 0.339, 0.345, and 0.856 pA. Thus, in guinea-pig heart there is no apparent tendency for the unitary currents to decrease when Ba replaces Ca. The linear conductances of guinea-pig L channels is 9.1 pS (10 Ca), 13.6 pS (7 Ca/3 Ba), and 20.7 pS (10 Ba), which agree closely with ours. We conclude that 10 Ca/1 Ba, as opposed to 10 Ca alone, would not significantly alter our estimate of conductance during the rare, long openings. Another possibility is that mode 2 kinetics in 10 Ca/1 Ba represents a temporary switch from Ca conduction to Ba conduction. According to Yue and Marban (1990), the conductance would be about right for 1 mM Ba. We exclude this prospect because independent evidence suggests that Ca should be the carrier in 10 Ca/1 Ba (Lansman et al., 1986), and because we see the long-duration currents (possibly contaminated by K currents) in the absence of Ba. Assuming that Yue and Marban's interpretation of the whole-cell mole fraction result holds, yet another possibility exists: mixtures of Ca and Ba may lower the current by changing the kinetics. Taking this view the average Ca current might actually be larger without Ba in the pipette. Such effects, however, are expected to be minor compared to the differences between 10 Ba and 10 Ca/1 Ba exemplified in Fig. 4, *c* and *d*.

The Ca conductance is practically impossible to define without the spontaneous long openings. Most often the currents are too brief, and no well-defined levels appear at any voltage. Increasing the bandwidth above 1,000 Hz makes the definition of conductance even more difficult, and it should be clear that our values are approximations limited to the conditions of the experiments. In Ca solutions, most patches apparently contain many channels whose openings cluster near the begnning of positive voltage steps. Within the same experiment, however, the patches appear to acquire a single, long-lasting conductance state sporadically. We interpret the long openings as kinetic conversions to mode 2. Like the Ba currents, Ca mode 2 currents display brief openings and closings as the voltage approaches −20 mV. Thus, once in mode 2 the Ca kinetics appear to follow the voltage. Unlike Ba, however, the Ca-conducting channel is less liable to present the long opening during the next beat. These observations

argue against the idea that mode 2 is a slow, possibly covalent transition of the channel. Instead, the dramatic difference between the normal Ba current and the normal Ca current would seem to reflect the ability of Ca ions to inhibit the Ca current, a capacity that is occasionally relaxed.

In the absence of exogenous stimulation, the only effect of mode 2 kinetics is a rounding of the action current as it descends from its peak value during repolarization (Fig. 4 *d*). Thus, even though they occur naturally in spontaneously beating cells, mode 2 kinetics are too rare to contribute much to the shape of the cardiac action potential. However, in the presence of stimulation that would promote mode 2, our experiments predict that the Ca action current (Fig. 4 *d*) would evolve into something similar to the Ba action current (Fig. 4 *c*). More charge would enter the cell during the plateau phase and the repolarization phase of the action potential. By this mechanism alone, stimulation of β-adrenergic pathways would prolong the action potential.

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