GATING OF DELAYED RECTIFICATION IN ACUTELY ISOLATED CANINE CARDIAC PURKINJE MYOCYTES

Evidence for a Single Voltage-gated Conductance

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ABSTRACT Studies of time-dependent, plateau outward current (delayed rectification) in the heart are complicated by the accumulation and depletion of K^+ ions in intercellular clefts. To minimize this problem, we studied delayed rectification in acutely isolated (enzymic solution, gentle agitation) canine cardiac Purkinje myocytes using the single microelectrode voltage-clamp technique. We found a sigmoidal voltage-dependence for activation of outward plateau current, with maximal activation occurring at potentials near −10 mV. The activation and deactivation of plateau outward current was adequately described as the sum of a fast and slow exponential component. A comparison of the time course of activation of plateau outward current and the "envelope" of tail currents suggests that a single voltage-gated conductance with one open and two closed states can account for delayed rectification in Purkinje myocytes. These results differ from those previously obtained with intact sheep Purkinje fibers in which two time-dependent conductances were postulated to account for delayed rectification (Noble, D., and R. W. Tsien, 1969, *J. Physiol. (Lond.)*, 200:205–231).

INTRODUCTION

Classical descriptions of the action potential of cardiac Purkinje fibers suggest that final repolarization commences when outwardly directed potassium current(s) activated during the plateau exceed in magnitude the inactivating inward calcium current (Noble, 1975; McAllister et al., 1975). Studies of these potassium currents (delayed rectification) are complicated by K^+ fluctuations in intercellular clefts that occur during activity (Kline et al., 1980) and during protocols used to study channel gating (Baumgarten and Isenberg, 1977; Levis et al., 1983; Kline and Cohen, 1984). These fluctuations contaminate kinetic measures of K^+ channel gating by altering the K^+ electrochemical gradient as well as channel conductance (Attwell and Cohen, 1979; Cohen and Kline, 1982). To avoid these difficulties, we reinvestigated the gating kinetics of delayed rectification in acutely dissociated canine cardiac Purkinje myocytes in which extracellular ion concentration fluctuations are reduced if not entirely eliminated (Mathias et al., 1985). Preliminary results have appeared in abstract form (Cohen et al., 1984; Datyner et al., 1984).

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METHODS

Adult mongrel dogs were euthanized with T-61 solution (National Laboratories Am-Hoechst Corp., Somerville NJ) and their hearts immediately removed. Free-running Purkinje fibers were excised from both ventricles, sliced, and placed in a dissociation solution containing (in millimoles per liter): KCl, 140; $NaHCO_3$, 8; NaH_2PO_4, 0.4; $MgCl_2$, 2; dextrose, 10; taurine, 25; β-hydroxybutyric acid, 5; Na-pyruvate, 5; (gassed with 95%:5% O_2/CO_2, pH 7.0, 20–40 μM Ca^{++} activity) along with collagenase (1.6–2.1 mg/ml Type II [Worthington Diagnostics, Freehold, NJ]) and bovine serum albumin (1.3–1.8 mg/ml, Sigma Chemical Co., St. Louis, MO). Fibers were triturated via their repeated passage through a pipette orifice (Datyner et al., 1985*a*). After three 20-min digestions, single cells were harvested and placed in a heated superfusion chamber (34–36°C [held within ±1.0°C in any individual experiment] [Datyner et al., 1985*b*]). Cells were superfused (1 ml/min) with a solution containing (in millimoles per liter): NaCl, 140; $MgCl_2$, 2.0; dextrose, 10; HEPES, 5; KCl, 8; $CaCl_2$, 2—or 1, where indicated (adjusted with 1 M HCl to pH 7.4) and viewed with Hoffmann modulation contrast optics (200 ×). Cells chosen for study were relaxed and rod-shaped, with prominent cross-striations; the typical lifespan of such cells in the superfusion chamber ranged from 3 to >6 h. Electron micrographs showed isolated myocytes without glycocalyx but with intact ultrastructure. No evidence was found for accumulation spaces from morphologic or impedance studies (Mathias et al., 1985).

Membrane currents were studied using single microelectrode (filled with 2.5 M KCl, 25–50 MΩ DC resistance) discontinuous voltage-clamp technique (switching frequency 1–2.5 kHz). Voltage and current traces were recorded on FM tape (3964a, 15/16 inches per second, 300 Hz bandwidth; Hewlett-Packard Co., Palo Alto, CA) for later display and analysis. Filtered currents (8 pole lowpass Bessel, cut-off frequency

indicated in figure legends) were digitized (12 bit resolution) and compared to fits generated using weighted least squares exponential regression.

RESULTS

Fig. 1 shows a sample action potential (inset) and steady-state current voltage relationship from a dissociated Purkinje myocyte. The action potential has a rapid upstroke (partially obscured by stimulus artifact), an overshoot, notch, and duration of 285 ms; however, the plateau occurs at more negative potentials than commonly seen with intact Purkinje fibers (see also Sheets et al., 1983). The steady-state current-voltage relationship contains regions of inward rectification, negative slope, and outward rectification and is similar to those obtained from canine Purkinje fibers (Cohen et al., 1983).

The steady-state activation curve for plateau outward current was determined by comparing the amplitudes of tail currents following prolonged depolarized pulses (see Fig. 2). There is a sigmoidal relationship between depolarized pulse potential and activation of plateau outward current, with maximal activation occurring at approximately −10 mV. Determination of the lower portion of the activation curve was made difficult by activation of the pacemaker current I_f (DiFrancesco, 1981). Determination of the reversal potential for the plateau outward current was difficult because of activation of I_f and the accelerated decay of tail current at more hyperpolarized potentials; single electrode-clamp technique posed further limitations by effectively limiting current-passing capabilities. However, in experiments in which the pacemaker and background currents were relatively small the reversal potential for plateau outward current was more negative than −60 mV ($[K^+]_o = 8$ mM). Plateau outward current was reduced by 1 mM $BaCl_2$, a known blocker of potassium

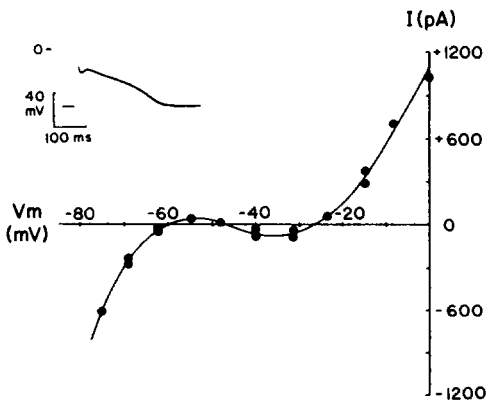

FIGURE 1 Current-voltage relationship of an acutely dissociated canine cardiac Purkinje myocyte. Membrane currents were plotted for 5-s voltage-clamp pulses to selected potentials from a holding potential of −62 mV. (*Inset*) Representative action potential from a Purkinje myocyte. Intracellular stimulation at 1 Hz. Maximum diastolic potential, −67 mV.

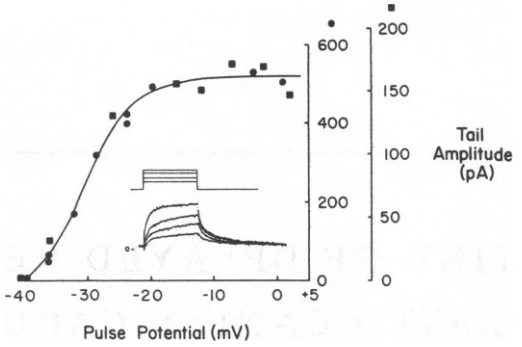

FIGURE 2 Determination of the voltage dependence for activation of plateau outward current. Tail current amplitudes were measured following 20-s depolarizing pulses to various potentials from a holding potential of −40 mV. Experimental results from two different myocytes are illustrated: the ordinates have been scaled to match the maximal tail current amplitude. $[Ca^{++}]_o$: ■, 1 mM; ●, 2 mM. (Inset) An example of currents during and after depolarizing pulses to −32, −28, −23, and −19 mV (from ●). Currents were filtered (6 Hz) and digitized before plotting. Holding current, +25 pA.

channels (Sperelakis et al., 1967; Standen and Stanfield, 1978; Yanigahara and Irisawa, 1980; Cohen et al., 1983).

We investigated the time course of activation and deactivation of plateau outward current. Fig. 3 *A* shows sample data obtained from a myocyte during a 20-s depolarizing pulse. The time course of membrane current is complex. The inward calcium current is seen in the first 10 ms. This current appears to activate and inactivate more rapidly in isolated Purkinje myocytes than in intact strand preparations (tau inactivation <10 ms [unpublished observations]; see also Isenberg and Klockner, 1982; Mitchell et al., 1983). Outward current does not attain a steady-state value during 20-s pulses, but continues to increase slightly ("creep") towards the end of the pulse. We found that outward current continues to slowly increase during depolarizing pulses as long as 50 s in duration, although >90% of the change in outward current is attained during the initial 15 s of such pulses. For this reason, we chose to analyze the plateau outward current recorded during 20-s pulses. The outward current "creep" was not investigated further. Transient outward current (which is known as I_{qr}, I_{co}, or I_{to}, as well as by other abbreviations [see Cohen et al., 1985]) was minimal during these studies. This small contamination could be due to partial inactivation of this current system at our chosen holding potentials, or to a genuine reduction in this conductance in our isolated cells.

Outward current during 20-s depolarizing pulses is well described as the sum of a fast and a slow exponential component (Fig. 3 *A*). Results similar to those presented were consistently obtained over the test potential range examined (−30 to 0 mV).

Fig. 3 *B* illustrates a tail current obtained following a 5-s depolarizing pulse. The time course of the tail current is well described as the sum of a fast and a slow exponential component. In all cases in which the tail currents were of

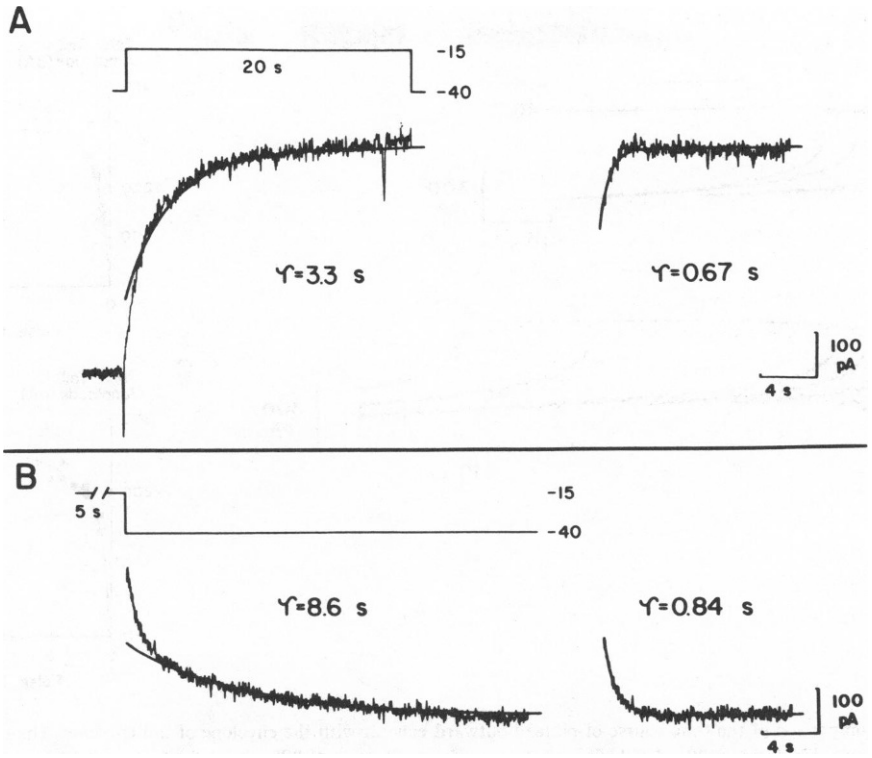

FIGURE 3 Plateau outward current recorded during and after depolarizing pulses were characterized as the sum of two exponential components. (*A*). Digitized currents recorded during a 20-s depolarizing pulse (*left*) were initially fit to an exponential with a time constant of 3.3 s (superimposed solid line). The difference between the superimposed fit and recorded current (*A, right*) was then fit to a second exponential with a time constant of 670 ms. (*B*). In a similar manner, tail currents were also characterized as the sum of two exponential components. In this example, the tail current following a 5-s depolarizing pulse (*B, left*) was fit to the sum of a slower ($\tilde{\tau}$ = 8.6 s) and a more rapid exponential (*B, right:* $\tilde{\tau}$ = 840 ms). Records sampled from FM tape at 20 Hz.

sufficient magnitude, the quality of the double exponential fit was comparable to that of Fig. 3 *B*.

To further study the kinetics of plateau outward current, we employed the "envelope test" (Hodgkin and Huxley, 1952; McAllister and Noble, 1966; Noble and Tsien, 1969; Carmeliet and Vereecke, 1979). According to this test, the time course of activation of a single conductance upon depolarization should be reflected in the growing "envelope" of peak tail currents obtained following progressively longer depolarizing pulses (see Fig. 4 *A*). If two independent, parallel conductances are present, the contribution of each conductance should be reflected in the growth of the envelope of tail currents.

Fig. 4 compares the time course of plateau outward current with the envelope of tail currents. Two approaches were used in constructing the envelopes. In Fig. 4 *A*, the envelope was constructed by measuring the heights of individual tail currents. In Fig. 4, *B* and *C*, each tail current was decomposed into two exponential components, and the envelope constructed by summing the amplitude of the fast and slow exponential components for each tail current. Using either approach, the envelope of tail currents describes the growth of plateau outward current (in six of six experiments).

The results presented in Fig. 4 are consistent with the plateau outward current resulting either from a single conductance with complex gating kinetics, or the sum of at least two conductances in parallel. To distinguish between these two possibilities, we performed envelope tests comparing the activation of the fast and slow components of plateau outward current with the growth of the corresponding tail current components (Fig. 5). The time course of activation of neither the fast nor the slow outward current component could describe the envelope of either the fast or slow component of tail currents. In all six completed tests, the envelope of the slow component of tail currents increased more rapidly than expected from the fit to the slow component of activating outward current. In five of these experiments, the envelope of the fast component of tail currents increased more slowly than did the fast component of the activating outward current. These results are inconsistent with the hypothesis that the two exponential components of plateau outward current result from the gating of two parallel conductances.

DISCUSSION

Our results demonstrate the following: (*a*) the biexponential relaxation of the delayed rectifier; (*b*) that the envelope

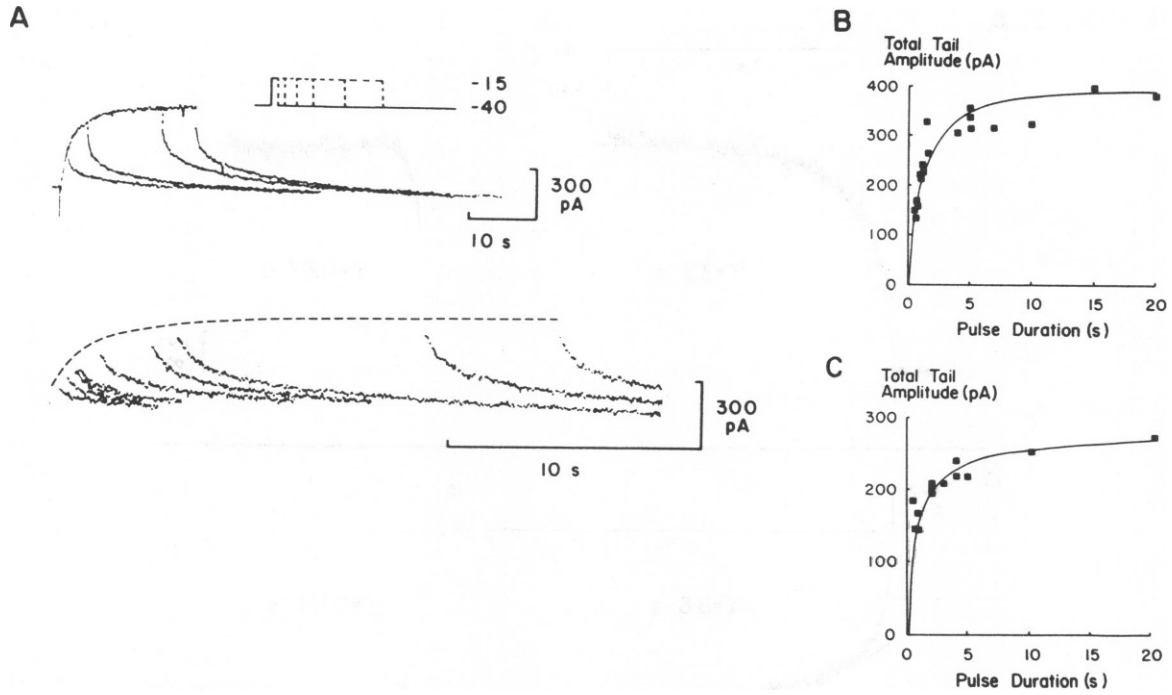

FIGURE 4 (*A*) A comparison of the time course of plateau outward current with the envelope of tail currents. The upper traces illustrate membrane currents recorded during a 20-s depolarizing pulse as well as tail currents following 1.5-, 4-, 15-, and 20-s pulses. The lower traces illustrate the initial portions of these and other tail currents on an expanded time scale. The dashed line represents outward current activated during the 20-s depolarizing pulse (scaled to ~75%). The envelope of tail currents parallels the growth of plateau outward current during depolarization. (*B* and *C*) Two experiments comparing the time course of plateau outward current with envelopes representing the summed amplitude of fast and slow components of individual tail currents. The envelope of summed tail current components describes the growth of outward current activated upon depolarization. *A* and *B* are from one experiment.

of tail currents describes the growth of plateau outward current; and (*c*) that the envelope of the fast and slow exponential components of tail currents do not describe the growth of either component of plateau outward current activated upon depolarization. Kinetic studies from various excitable preparations suggest that many voltage-gated channels may have only one open state but more than one closed state. We considered this possibility to explain our observations of the cardiac delayed rectifier. The simplest kinetic schema consistent with a two closed state model is

$$C_1 \underset{k_{-2}}{\overset{k_2}{\longleftrightarrow}} C_2 \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} O, \qquad (1)$$

where C_1 and C_2 represent the probability of being in either of two closed states, O represents the probability of being in the open state, and k_1, k_{-1}, k_2, and k_{-2} are voltage-dependent rate constants. The time course and magnitude of changes in O following a step change in potential is

$$O(t) = R_1 \exp(-t/\tau_1) + R_2 \exp(-t/\tau_2) + O_\infty, \qquad (2)$$

where τ_1, τ_2, R_1 and R_2 are functions of voltage. θ_1 and θ_2 are determined entirely by the voltage-dependent rate constants and R_1 and R_2 depend upon both the rate constants and the initial conditions (see Colquhoun and Hawkes, 1981). From Eq. 2, it can be seen that the relaxation of outward current upon a step change in potential is described by the sum of two exponentials, while the time course of activation of outward current should be reflected in the amplitude of tail currents measured upon repolarization. Both predictions are consistent with our experimental findings.

Voltage-clamp studies using intact sheep Purkinje fibers suggest that two time-dependent outward potassium currents (I_{x1} and I_{x2}) activated in the plateau range of potentials could account for delayed rectification (the parallel conductance hypothesis, Noble and Tsien, 1969). Our results are similar to those from sheep fibers in that (*a*) slow outward membrane current is present in the plateau range of potentials, as is (*b*) the biexponential relaxation of outward membrane current following step changes in membrane potential. Our results are dissimilar to those from sheep fibers in that maximal activation of the delayed rectifier in dissociated myocytes is attained at potentials more negative than those obtained in intact Purkinje preparations (−10 vs. +20 mV). Noble and Tsien (1969) also found an inflection in the steady-state activation curve for the delayed rectifier. This inflection was postulated to result from the overlapping voltage ranges from activation of I_{x1} and I_{x2} and was therefore consistent with the parallel conductance hypothesis. We find no such inflection with dissociated myocytes. The

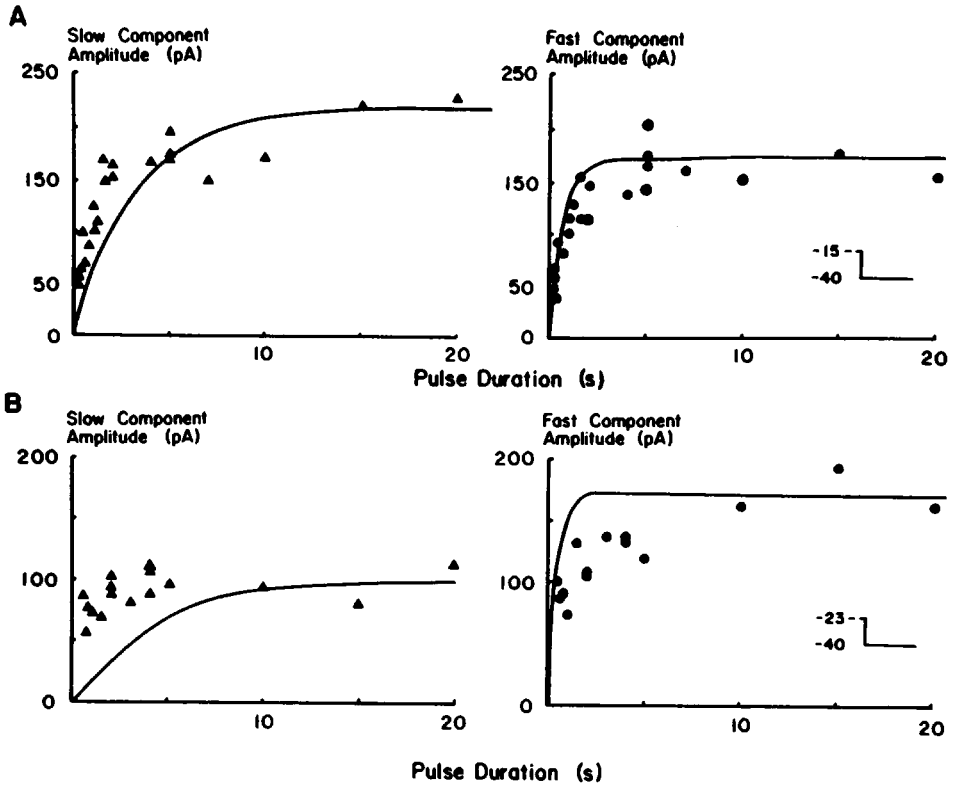

FIGURE 5 A comparison of the activation of fast and slow components of plateau outward current with the envelope of corresponding tail current components. For each panel, triangles (*left*) represent the amplitude of the slow component of tail currents, while circles (*right*) represent the amplitude of the fast component of the tail currents. The exponential curves on each graph represent exponential fits to the corresponding rapid and slow components of plateau outward current activated during 20-s depolarizing pulses. These fits were linearly scaled so as to asymptote with the corresponding maximum tail current component obtained during 20-s pulses (as judged by eye). The envelopes of neither the fast nor slow components of tail currents describe the growth of the corresponding outward current component activated upon depolarization. Fits also were sub-optimal when displayed on semilogarithmic coordinates. Voltage-pulse protocol for both experiments indicated in each panel. *A* is from the same experiment as Fig. 4 *B*, and *B* from the same experiment as Fig. 4 *C*.

parallel conductance hypothesis fails in isolated Purkinje myocytes.

The contrasting results in strand vs. myocyte preparations may be related to distortions in K^+-channel gating introduced by fluctuating cleft K^+ concentrations in intact strand preparations that are absent in dissociated Purkinje myocytes. Alternatively, these disparate results may be due to differences in the conductances responsible for plateau outward current in the two preparations. Arguing against this latter possibility is the observation that selectively studies in intact calf Purkinje fibers suggest that delayed rectification can be accounted for by a single population of channels that exist in more than two states (Bennett et al., 1984).

We have presented an alternative hypothesis in which delayed rectification is ascribed not to two parallel conductances in the cardiac membrane, but rather to the existence of a single conductance with at least two closed states but one open state. We have provided a first test of this hypothesis by demonstrating that the predictions of such a model qualitatively agree with experimental results in dissociated Purkinje myocytes. In light of this new interpretation, the kinetics of delayed rectification and the effects of drugs and neurohumors on this conductance mechanism may require reevaluation.

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