Brief Communication

Reconstitution and Characterization of a Calcium-Activated Channel From Heart

Joseph A. Hill Jr., Roberto Coronado, and Harold C. Strauss

This paper is the first description of a calcium-activated nonspecific cation channel in adult ventricular muscle. We report gating kinetics and ionic selectivity data from experiments performed at the single channel level. Calcium activation is described by a gating model wherein two ions are involved in the reaction. Channel gating exhibited marked voltage dependence. Ionic selectivity experiments indicated that the channel is cation-selective but unable to discriminate between Na\(^+\) and K\(^+\). We discuss evidence that this channel mediates transient inward current in ventricular tissue; thus, the channel may be involved in afterdepolarization-induced cardiac arrhythmias. (Circulation Research 1988;62:411-415)

The several effects of calcium on cardiac electrical properties are the subject of intense interest at present. For example, an oscillatory inward current exists in calcium-overloaded cardiac muscle following depolarizing voltage-clamp steps.\(^1\),\(^2\) At present, the molecular mechanism that underlies this transient inward current (I\(_\text{inward}\)) remains unidentified. A substantial body of literature supports the hypothesis that this current is calcium-induced\(^3\),\(^4\) and involved in the genesis of afterdepolarizations and aftercontractions.\(^1\),\(^3\) In addition, it is known that afterdepolarizations can trigger extra beats in isolated cardiac preparations\(^5\) that sometimes develop into runs of repetitive activity. Thus, I\(_\text{inward}\) may be an important pathological mechanism underlying the clinical events accompanying calcium overload.

Channel opening gives rise to an elementary current pulse, the size and duration of which can be observed directly by using single-channel recording techniques.\(^6\) One such technique, planar bilayer reconstitution, involves the incorporation of native channel protein into an artificial membrane with simultaneous measurement of transmembrane current at high gain. Using this technique, we describe a calcium-activated channel from cardiac ventricular sarcolemma that would mediate inward current under physiological conditions. As such, these data support the hypothesis that channel-mediated current plays a role in I\(_\text{inward}\) and in the development of afterdepolarizations and triggered arrhythmias.

Materials and Methods

Sarcolemmal vesicles from adult canine left ventricle were prepared according to the method of Jones.\(^7\) Lipid bilayers were prepared by painting a 1:1 (wt/wt) phospholipid mixture of phosphatidylethanolamine and phosphatidylserine (Avanti Polar Lipids, Birmingham, Alabama) dissolved in decane (20 mg lipid/ml decane) across a 300-μm diameter aperture separating two aqueous chambers (cis, trans). Typical bilayers exhibited 200-300 pF capacitance. Resistances were always greater than 100 GΩ. The ventricular vesicles were incorporated into the artificial membrane by adding them to the cis chamber under fusion conditions.\(^8\) All aqueous solutions were buffered with 10 mM histidine (pH 7.1). Bath calcium activity was calculated in CaEGTA solutions using an effective stability constant of 4.31×10\(^7\) M\(^{-1}\) (0.1 M ionic strength, pH 7.1, 25\(^\circ\) C).\(^9\) All experiments were performed at room temperature (25-26\(^\circ\) C) and under steady-state conditions. The cis chamber was connected to a voltage source while the trans chamber was held at virtual ground by a current-to-voltage converter circuit. Analog data were low-pass filtered (usually −3 dB at 100 Hz, eight-pole Bessel) and stored on FM tape. Later, the signal was sampled and stored on magnetic disk for digital analysis. Linear regression was performed according to the method of least squares. Abbreviations used include: [X], concentration of species X; KOAc, potassium acetate; EGTA, ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid; pCa, −log([Ca]).

Results

Gating Kinetics

A representative continuous record of single channel current is illustrated in Figure 1A. As can be seen,
gating occurs in bursts of activity separated by relatively long periods of silence. The open state (displayed up) exhibits flickery kinetics as it is interrupted by numerous short-lived closures. In all cases, distributions of open channel duration or closed state duration were fitted satisfactorily only by the sum of two exponential components (Panel B).

Single channel open probability is modulated by cis calcium activity (Figure 2). Representative recordings selected from data points depicted in Panel B are shown in Panel A. Open probability ranges from 8% at 0.2 μM Ca\(^{2+}\) in the cis chamber to 99% at 10 mM Ca\(^{2+}\); this effect is readily reversible upon the addition of EGTA. Popen, a function of cis calcium concentration defined as cumulative open time divided by the sum of cumulative open and closed times \([O/(O + C)]\), varied approximately between unity and 0 over the pCa range of 6 to 7 (Panel B). This observation fits well with the experimentally observed values of intracellular calcium in ventricular myocytes under physiological conditions (approximately 100 nM diastolic Ca\(^{2+}\)) and in states of calcium overload (1 μM and above).\(^1\) We have, however, observed shifts in the open state probability between different experiments (cf., Figure 2A and Figure 3) as have other investigators working with calcium-activated potassium channels.

The sigmoidal curve superimposed on the data in Figure 2B is that predicted by a model wherein two calcium ions bind to the channel. A fit to the data was obtained by plotting \(1/P_{\text{open}}\) versus \([Ca^{2+}]^{-2}\) (Panel C). The slope of the fitted line was \(3.1 \times 10^{-4}\) M\(^{-2}\) and is a measure of the equilibrium dissociation constant, \(K_d\). In all experiments (n = 9), the calcium-sensitive site was oriented toward the cis chamber.

Methfessel and Boheim have proposed an activation/blockade model to describe the gating kinetics observed in calcium-activated channels from skeletal muscle:

\[
\text{Ca}^{2+} + \text{R} \rightleftharpoons \text{R}-\text{Ca} \rightleftharpoons \text{R}^*-\text{Ca} \rightleftharpoons \text{R}^*-\text{Ca}^2+, (1)
\]

where R is the channel receptor, and the asterisk signifies the open state. This linear scheme is a hybrid of two models, viz., an activation model in which a calcium ion binds to the closed channel allowing it to open (\(R \leftarrow \rightarrow R-Ca \leftarrow \rightarrow R^*-Ca\)) and a “blockade” model in which a second calcium ion stabilizes the open configuration (\(R-Ca \leftarrow \rightarrow R^*-Ca \leftarrow \rightarrow R^*-Ca_2\)).

Scheme 1 allowed us to make several predictions for experimentally observable variables. First, the model predicts that except under limiting conditions (very high or very low \([Ca^{2+}]\)), two exponential components will be required to describe the open and closed state probability distributions (Figure 1B). Second, the model predicts that the ratio of mean open time to mean closed time (equilibrium constant, \(K\)) will be a function of calcium concentration raised to an exponent reflecting the number of ions that bind to the channel. A best-fit line obtained when these data are plotted as \(\log(K)\) versus \(\log([Ca^{2+}]^2)\) had a slope of 2.5 (data not shown). Third, scheme 1 predicts that under limiting conditions of calcium activity, mean open time is a linear function of \([Ca^{2+}]\) and that mean closed time is...
Hill et al  Cardiac Calcium-Activated Channel

Figure 2.  Popen is a function of cis calcium concentration. Panel A, Representative records are shown from data points in Panel B. Popen [defined as cumulative open time divided by the sum of cumulative open and closed times, O/(O + C)] is as listed; symmetrical [KoAc] = 100 mM, cis [Ca2+] = 10 mM, 1.6 µM, 0.4 µM, 0.25 µM, and 0.2 µM, trans [Ca2+] < 100 nM (open = up). Panel B, Popen is plotted versus $-\log([Ca^{2+}])$. All recordings were made at −40 mV holding potential. The continuous curve is the transformed equation from the analysis in Panel C. Panel C, 1/Popen versus 1/[Ca2+]. Coefficient of correlation ($r$) of the fitted line is 0.988. Panel D, Open and closed state durations are functions of cis [Ca2+]. $\bullet$, log mean open time versus log([Ca2+]). The slope of the best fit line is 0.93, $r = 0.93$; $\circ$, log mean closed time versus log([Ca2+]). The slope of the best fit line is $-1.5$, $r = 0.90$.

Channel gating was strongly voltage-dependent (Figure 3). In this experiment, cis and trans calcium concentrations are symmetrical and represent base line calcium for solutions in our laboratory in which neither calcium nor chelator is added. We have subsequently measured this calcium concentration as less than 100 nM. Single channel open probability varied from approximately 0 to 1 over the voltage range −60 mV to 0 mV under these conditions. The sigmoidal activation curve depicted is a Boltzmann relation obtained as a fit to the logit transformation $\ln\left(\frac{1 - P\text{open}}{P\text{open}}\right)$ versus voltage. The slope of the fitted line is interpreted to indicate that −2.5 gating charges move from trans to cis upon channel opening. The y intercept obtained by this method indicates that −2.2 kcal/mol "nonelectrical" free energy are required for channel opening. That this nonelectrical component is less than 0 confirms that the open state is favored at 0 mV.

Ionic Selectivity

Figure 4 depicts single channel current as a function of holding voltage. Each data point represents the mean of at least three (and usually four to seven) amplitude measurements. Single channel current was ohmic over the voltage range −80 to +20 mV. In the presence of a KCl activity gradient across the bilayer of 1.4, the interpolated reversal potential was −7 mV, which agrees well with the Nernst potential for K+ (−9 mV) (Panel A). In the presence of increasingly asymmetric KCl solutions, the single channel current-voltage relation shifts toward more negative potentials. In each case, the experimentally determined reversal potential agreed with the Nernst potential for K+ within 3 mV. Thus, we conclude that the channel selects strongly for cations over anions.

We have also measured the reversal potential under bionic conditions for Na+ and K+ (Figure 4B). A fitted line is superimposed on the data that has an x intercept of +2 mV and a slope of 120 pS. By assuming ionic independence, we may invoke the Goldman-Hodgkin-Katz equation for bionic conditions to calculate relative ionic permeabilities. In so doing, it is clear that these data are consistent with a channel that is completely nonselective between Na+ and K+ ions. This observation suggests that the channel conducts current that reverses near 0 mV under physiological...
conditions. We interpret the conductance (120 pS) to represent a first approximation (neglecting temperature effects) to the channel's conductance in a physiological environment.

At present, we are unable to quantify relative calcium permeability. However, the experiment shown in Figure 2 allowed us to estimate relative Ca$^{2+}$ permeability with respect to K$^+$ at less than 0.2. Single channel current increased less than 0.7 pA (5%) at $-40$ mV over the pCa range of 2 to 7 in the cis chamber; from this, we conclude that the channel exhibited relatively little calcium permeability.

Discussion

Calcium-Activated Channels in Other Tissues

This report represents the first description of single calcium-activated channels from working adult myocardium. Colquhoun et al. have described a calcium-activated channel from cultured neonatal rat heart that exhibited some of these same characteristics except that the single channel conductance was markedly lower, viz., 30–40 pS in the presence of physiological salt solutions. In addition, they observed voltage-independent gating and calcium sensitivity in the 1–10 $\mu$M range. We speculate such discrepancies may be due to species and tissue development differences. One report has been published on single channel measurements of a calcium-activated potassium-selective channel in bovine cardiac Purkinje fibers. Calcium-activated channels have also been described from skeletal and cardiac sarcoplasmic reticulum. The sarcoplasmic reticulum channels, however, differ from the one described here in several respects: 1) they select for divalent cations over monovalents by a factor of 10, 2) are weakly voltage-dependent, 3) display open lifetime kinetics that are independent of calcium concentration, 4) have a calcium binding stoichiometry of 0.3 (R. Coronado, personal communication) instead of 2 (Figure 2C), and 5) are inhibited by cis calcium concentrations above 1 mM unlike activity as shown in Figure 2A.

Most of the biophysical measurements we report here are similar to those reported by others studying calcium-activated channels. Specifically, channel gating displays bursting kinetics, a characteristic feature of calcium-activated potassium channels from skeletal muscle as well as calcium-activated nonspecific cation channels from cultured neonatal rat hearts. Indeed, it was the distinctive appearance of the open state that led us to investigate the possibility of calcium-dependent gating. Second, large conductance is characteristic of calcium-activated channels, with a few notable exceptions, such as the calcium-activated channel of neonatal rat heart (see above). Marked voltage dependence of gating and an agonist-receptor stoichiometry of 2:1 are features of calcium-activated channels in skeletal muscle. In fact, the binding of two agonist ions for channel activation is in keeping with the general properties of many ligand-gated channels, e.g., calcium-activated channels, acetylcholine receptor channels, and glycine and $\gamma$-aminobutyric acid receptors. Finally, the calcium dependence of mean open time and mean closed time is similar to that observed in calcium-activated potassium channels from cultured skeletal muscle studied with the patch-clamp technique or reconstituted into bilayers.

Significance

We were able to reconstitute this channel into bilayers sporadically; this suggests that the channel may be present in the preparation in relatively small numbers. Further, the fact that the cardiac channel has never been observed with the patch-clamp technique...
suggested that this channel may be localized to clefts in the sarcosommal membrane as in skeletal muscle. However, in order to elucidate further its physiological role, it will be necessary to demonstrate its function directly using either patch clamping or high-affinity ligand binding techniques. In planning such experiments, it is a useful exercise to estimate channel density.

We have estimated the channel’s conductance in a physiological environment at 120 pS (see above). In addition, we have assumed 100 nA of transient inward current in a Purkinje fiber strand of radius 100 μm and length 1,000 μm (see Cannell and Lederer, Figure 1). Finally, true membrane surface area was taken to be 10 times the apparent surface area of a smooth envelope of membrane.26 Macroscopic current I is given by

\[ I = n i p, \]

where \( n \) is number of channels, \( i \) is single channel current, and \( p \) is channel opening probability (taken to be \( 1 \) at 1 μM calcium and \(-50 \) mV, Figure 1A). Further, \( i = v \gamma \), where \( v \) is electrochemical driving force (taken to be 50 mV), and \( \gamma \) is single channel conductance. With these assumptions, we have estimated the channel density to be 1 per 200 μm². Thus, we predict that if this channel mediates \( L_\alpha \), then it will be sparsely distributed in the cell membrane.

Cannell and Lederer suggested that the transient inward current in sheep Purkinje fibers is dependent on channel current (although Na-Ca exchange–mediated current or current from other electrogenic mechanisms may also be involved). The putative transient inward channel must exhibit calcium-dependent gating over a physiological range of intracellular calcium, conduct inward current at diastolic membrane potentials, and select for cations over anions. Our measurements of calcium sensitivity, \( P_m, P_x \) approaching 1, and chloride impermeability are consistent with these predictions. Further, the steep voltage dependence we observed may explain the characteristic curvature in the macroscopic current-voltage relation.3 We have not tried to measure \( Ca^{2+} \) current under the same conditions used by these investigators (replacing \( trans \) monovalent cations with \( Ca^{2+} \)); we do report, however, absence of substantial calcium permeability in the presence of monovalent cations. This observation is consistent with studies of calcium-activated channels from neuroblastoma.27 For these reasons, we propose that this channel may contribute to transient inward current in canine ventricular muscle and may be involved in the development of triggered arrhythmias in calcium-overloaded tissue.

Acknowledgments
We gratefully acknowledge Dr. Jonathan Lederer for his critical reading of the manuscript. We thank Joseph C. Greenfield Jr. for continued support and S. Webb and B. Hill for editorial assistance.

References

Key Words: reconstitution • calcium-activated channel • triggered arrhythmia
Reconstitution and characterization of a calcium-activated channel from heart.
J A Hill, Jr, R Coronado and H C Strauss

doi: 10.1161/01.RES.62.2.411

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/62/2/411

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/