Activation of Single Cardiac and Skeletal Ryanodine Receptor Channels by Flash Photolysis of Caged Ca^{2+}

Sandor Györke, Patricio Vélez, Benjamín Suárez-Isla,* and Michael Fill

Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77555-0641 USA, and *Departamento de Fisiologia y Biofisica, Universidad de Chile, Facultad de Medicina, Casilla 70005, Santiago 7, Chile

ABSTRACT Single ryanodine-sensitive sarcoplasmic reticulum (SR) Ca^{2+} release channels isolated from rabbit skeletal and canine cardiac muscle were reconstituted in planar lipid bilayers. Single channel activity was measured in simple solutions (no ATP or Mg^{2+}) with 250 mM symmetrical Cs^+ as charge carrier. A laser flash was used to photolyze caged-Ca^{2+} (DM-nitrophen) in a small volume directly in front of the bilayer. The free [Ca^{2+}] in this small volume and in the bulk solution was monitored with Ca^{2+} electrodes. This setup allowed fast, calibrated free [Ca^{2+}] stimuli to be applied repetitively to single SR Ca^{2+} release channels. A standard photolytically induced free [Ca^{2+}] step (pCa 7→6) was applied to both the cardiac and skeletal release channels. The rate of channel activation was determined by fitting a single exponential to ensemble currents generated from at least 50 single channel sweeps. The time constants of activation were 1.43 ± 0.65 ms (mean ± SD; $n = 5$) and 1.28 ± 0.61 ms ($n = 5$) for cardiac and skeletal channels, respectively. This study presents a method for defining the fast Ca^{2+} regulation kinetics of single SR Ca^{2+} release channels and shows that the activation rate of skeletal SR Ca^{2+} release channels is consistent with a role for CICR in skeletal muscle excitation-contraction coupling.

INTRODUCTION

Specialized Ca^{2+} channels (ryanodine receptors, RyRs) mediate the release of Ca^{2+} from the sarcoplasmic reticulum (SR), an intracellular Ca^{2+} storage and release organelle (Endo, 1977; Meissner et al., 1986; Smith et al., 1988). In cardiac muscle, SR Ca^{2+} release is induced by Ca^{2+} which enters the cell through voltage-dependent Ca^{2+} channels in the surface membrane (Cleemann and Morad, 1991; Sipido and Wier, 1991). This mechanism is termed Ca^{2+}-induced Ca^{2+} release (CICR) (Fabiato, 1983, 1985; Ford and Podolsky, 1970). In contrast, it is clear that Ca^{2+} influx through the surface membrane is not required for activation of Ca^{2+} release in skeletal muscle (Armstrong et al., 1972; Spiecker and Luttgau, 1979). Instead, a direct communication between a surface membrane (T-tubule) voltage sensor protein and the skeletal SR Ca^{2+} release channel has been proposed to mediate the signal transduction (Rios et al., 1991; Rios and Pizarro, 1991). However, the role (if any) of CICR in skeletal muscle is still debated. It has been proposed that an initial "Ca^{2+} trigger" arises from T-tubule bound stores (Frank, 1980) or that CICR is a secondary amplification mechanism (Györke and Palade, 1993; Rios and Pizarro, 1991).

The single channel properties of SR Ca^{2+} release channels have been studied by reconstituting isolated channels into planar lipid bilayers (Chu et al., 1993; Lai et al., 1988; Rousseau and Meissner, 1989; Smith et al., 1986, 1988). In these studies, the action of Ca^{2+} has been established under steady-state conditions (Chu et al., 1993; Rousseau and Meissner, 1989; Smith et al., 1986). Maximal channel activation of both skeletal and cardiac channels occurs when the steady-state free [Ca^{2+}] is between 10 and 100 μM. These steady-state experiments, however, do not reveal if the kinetics of single channel Ca^{2+} regulation are physiologically appropriate. To play a significant role in excitation-contraction (E-C) coupling, Ca^{2+} activation of the channel should be at least as rapid as the rise of intracellular [Ca^{2+}] upon excitation, which occurs within ≈15 ms in skeletal muscle (Vergara and Delay, 1986) and within ≈25 ms in cardiac muscle (Cleenmann and Morad, 1991).

To determine the Ca^{2+} activation rate of single SR Ca^{2+} release channels, flash photolysis of caged-Ca^{2+} was used to apply fast calibrated free [Ca^{2+}] stimuli to channels reconstituted in planar bilayers. Recently, this method was used to demonstrate that single cardiac SR Ca^{2+} release channels adapt to sustained Ca^{2+} steps (Györke and Fill, 1993). In this paper, details of the flash photolysis methodology are presented for the first time. Further, we show that a standard fast free [Ca^{2+}] stimuli (pCa 7→6) activates the cardiac and skeletal release channels equally fast. Thus, the Ca^{2+} activation rate of single skeletal SR Ca^{2+} release channels is adequate to be consistent with a role for CICR in skeletal muscle E-C coupling.

MATERIALS AND METHODS

Heavy SR microsomes were isolated from canine ventricular muscle and rabbit skeletal muscle using established techniques (Tate et al., 1985). Briefly, the muscle was collected, cut into small pieces, and homogenized. Differential centrifugation was used to separate heavy microsomal fractions. Heavy SR microsomes were isolated on a discontinuous sucrose gradient. Microsomal fractions were stored at −80°C until used.

Single SR Ca^{2+} release channels were reconstituted by fusing heavy SR microsomes into planar lipid bilayers. Fusion was promoted by an osmotic gradient across the bilayer and evident by the sudden appearance of single-channel activity. Once a channel was reconstituted, the probability of fusion

Received for publication 22 November 1993 and in final form 16 February 1994.

Address reprint requests to Dr. Michael D. Fill, Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, TX 77555-0641. Tel.: 409-772-1826; Fax: 409-772-3381.

0006-3495/94/06/1879/08 $2.00

was immediately reduced by eliminating the osmotic gradient. Planar bilayers were cast across a 150 μm diameter aperture in a Delrin partition. Bilayer-forming solution contained a mixture of phosphatidylethanolamine and phosphatidylcholine (7:3, 50 mg/ml decane) obtained from Avanti Polar Lipids (Pelham, AL). Heavy SR microsomes were applied to one side of the bilayer (defined as *cis*). The other side was defined as *trans*. Reconstituted channels were consistently oriented with the cytoplasmic channel face on *cis* side of bilayer (Fill et al., 1990). Solutions contained 250 mM $CsCH_3SO_3$ and 20 mM HEPES Tris (pH 7.4). In steady-state experiments, the free $[Ca^{2+}]$ was adjusted with EGTA (0.1→1.0 mM) and continuously monitored by a Ca^{2+} electrode. After DM-nitrophen (3 mM; Calbiochem, San Diego, CA) was added to the *cis* chamber, the free $[Ca^{2+}]$ was carefully titrated to exactly pCa 7. All chemicals were the highest quality available.

Single-channel activity was measured using a custom-designed amplifier (Fill et al., 1990) and a TL-1 AD/DA interface (Axon Instruments, Burlingame, CA). Data were sampled at 5–10 kHz and filtered at 3–4 kHz and stored on Bernoulli disk (Iomega, Roy, Utah). Single-channel analysis was performed using pClamp software (Axon Instruments, Burlingame, CA).

To photolyze caged-Ca^{2+} in the microenvironment of the reconstituted channel, an intense UV flash was applied by a frequency-tripled, Q-switched, Nd:yittrium-aluminum-garnet (YAG) laser. The UV flash was directed at the solution directly in front of the bilayer through a micropositioned single fused silica optic fiber (450 μm diameter). The free $[Ca^{2+}]$ directly in front of the bilayer was measured using a specialized Ca^{2+} electrode (see Fig. 2).

RESULTS

To define the time course of Ca^{2+} activation of single SR Ca^{2+} release channels required that repetitive, calibrated free $[Ca^{2+}]$ stimuli be applied by flash photolysis of caged-Ca^{2+}. The required modifications to the standard planar bilayer apparatus are diagrammed in Fig. 1. An intense UV flash from a laser was applied through a single optic fiber positioned in front of the bilayer.

Reproducible accurate physical positioning of the optic fiber was critical. The optic fiber was held perpendicular to the plane of the bilayer by a three-dimensional micromanipulator. Before casting the bilayer, visible laser light passing through the optic fiber was used to adjust its *x*- and *y* axis positions. The incident light was centered on the open aperture. A centered (*x*-*y*) optic fiber projects an intense light through the open aperture producing a bright light spot on the back wall of the Delrin cup. After casting the bilayer, the *z* axis position was adjusted using a stereo microscope so that the optic fiber was exactly 150 μm from its surface.

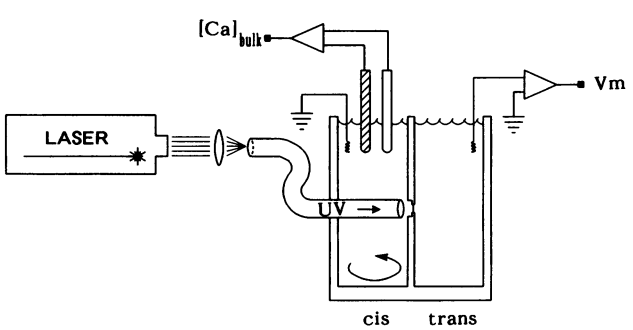

FIGURE 1 Schematic representation of the experimental setup for application of caged-Ca^{2+} methodology to single channels incorporated in lipid bilayers. The output of a Nd YAG laser was focused with a fused silica lens (focal length 3 cm) on the end of a 35 cm long, 450 μm diameter fused silica fiber optic. The other end of the fiber optic was positioned with a micromanipulator 150 μm in front of the bilayer. The free $[Ca^{2+}]$ in the bulk solution on the *cis* side of the bilayer was continuously monitored with a Ca^{2+} electrode (bulk electrode). Local $[Ca^{2+}]$ changes near the bilayer surface were calibrated by transforming the bilayer aperture into a Ca^{2+} electrode (local electrode) by filling it with Ca^{2+} ionophore resin mixture.

Photolysis of caged-Ca^{2+} (DM-nitrophen) occurred only in the small volume between the end of the optic fiber and the bilayer. The photolyzed solution in this small volume was replaced by unphotolyzed solution by rapidly stirring the bath. The free $[Ca^{2+}]$ in the bath was continuously monitored by a macroscopic Ca^{2+} electrode, the bulk Ca^{2+} electrode. The free $[Ca^{2+}]$ at the bilayer surface was measured by transforming the bilayer aperture into a Ca^{2+} electrode, the local Ca^{2+} electrode.

The local electrode and the bilayer were formed in the same aperture, although not simultaneously. First, channels were recorded in a bilayer and a set flash protocol was applied. After the experiment, the bilayer was broken and the same aperture was filled with a standard liquid ionophore resin from *inside* the Delrin cup. The solution inside the cup was then replaced with an electrode filling solution (100 mM $CaCl_2$ standard; Orion, Boston, MA). Thus, the same aperture was transformed into a Ca^{2+}-sensitive electrode without altering the relative positions of the Delrin cup and optic fiber. The same flash protocol was then applied to the resin-filled aperture, the local electrode. Additionally, control experiments using separate identical Delrin cups fit with permanent Ca^{2+}-sensitive membranes were performed. The optic fiber was micropositioned as described above, and the photolytic $[Ca^{2+}]$ changes induced by set flash protocols were calibrated.

The bulk and local Ca^{2+} electrodes were calibrated using Ca^{2+} standard solutions (World Precision Instruments, Sarasota, FL). Steady-state calibration curves measured simultaneously with the bulk Ca^{2+} electrode (*open circles*) and the local Ca^{2+} electrode (*filled circles*) are shown in Fig. 2 *A*. The line (slope = 27 mV/decade) was fit to the local Ca^{2+} electrode data.

Photolytically induced free $[Ca^{2+}]$ changes monitored by the local Ca^{2+} electrode are also shown in Fig. 2. In Fig. 2 *B*, 3 mM DM-nitrophen was added to the *cis* solution, and the resting free $[Ca^{2+}]$ was carefully titrated to pCa 7. Laser flashes of different intensities induced proportional free $[Ca^{2+}]$ elevations. The local $[Ca^{2+}]$ elevation induced by the flash was not detected by the bulk Ca^{2+} electrode. In a different experiment (Fig. 2 *C*), 1 mM Diazo-2 (caged Ca^{2+} chelator) was added, and the resting $[Ca^{2+}]$ was carefully titrated to 20 μM using the bulk Ca^{2+} electrode. Flash photolysis induced a step-like $[Ca^{2+}]$ decrease in our system. Thus, the local Ca^{2+} electrode was able to detect $[Ca^{2+}]$ changes in both directions. The local Ca^{2+} electrode data (Fig. 2, *B* and *C*) indicate that local free $[Ca^{2+}]$ remains constant for several seconds after photolysis. The diffusion pathway out of the small region between the optic fiber and the bilayer is relatively long. Thus, it was not surprising that $[Ca^{2+}]$ remained constant for several seconds after a flash.

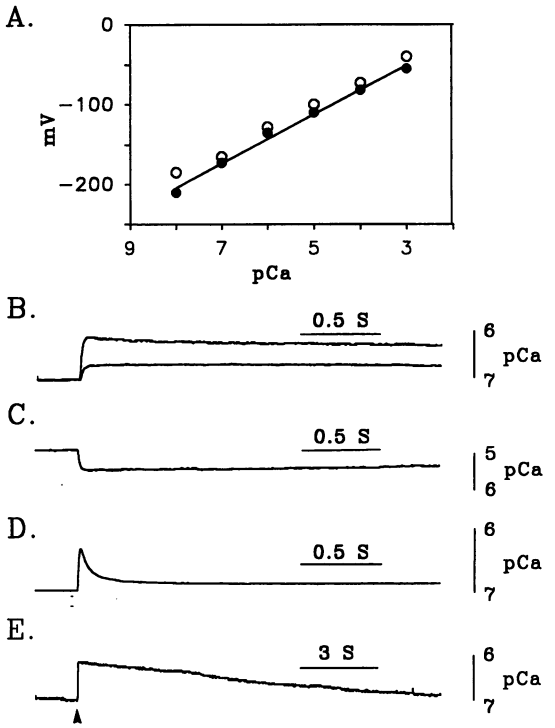

FIGURE 2 Calibration of the Ca^{2+} stimuli. (*A*) Steady-state calibration curves of the bulk Ca^{2+} electrode (○) and the local Ca^{2+} electrode (●) obtained with standard Ca^{2+} solutions. The line was fit to the local Ca^{2+} electrode data. (*B–E*) Photolytically induced changes in $[Ca^{2+}]$ measured with the local Ca^{2+} electrode under various experimental conditions. (*B*) Rapid and sustained $[Ca^{2+}]$ elevations induced by flashes of different intensity in the presence of 3 mM DM-nitrophen (initial $[Ca^{2+}]$ = 100 nM). (*C*) When DM-nitrophen was replaced with Diazo-2 (2 mM; photolabile Ca^{2+} chelator), a flash induces a step decrease in $[Ca^{2+}]$ (initial $[Ca^{2+}]$ = 20 μM). (*D*) Time course of the free $[Ca^{2+}]$ change in the presence of EGTA (1 mM) and DM-nitrophen (3 mM). Initial free $[Ca^{2+}]$ was 100 nM. (*E*) Time course of the free $[Ca^{2+}]$ change after a laser flash during continuous stirring of the bath. Initially, solution contained DM-nitrophen (3 mM) buffering Ca^{2+} at 100 nM.

The UV flash (10 ns duration) was electronically triggered. The variability in the timing of the UV flash was undetectable. The half-time of liberation from DM-nitrophen is faster than 180 μs (McCray et al., 1992). Because the local Ca^{2+} electrode responded with a time constant of 50–100 ms, it could not tract the relatively fast free $[Ca^{2+}]$ changes induced by photolysis of DM-nitrophen. One study that combined UV laser photolysis of DM-nitrophen while monitoring a fast fluorescent indicator revealed that Ca^{2+} was liberated with a time constant of 30 μs (Vergara and Escobar, 1993). In our system, therefore, we assume that Ca^{2+} was liberated at a similar rate.

To demonstrate that the local Ca^{2+} electrode can track slow transient $[Ca^{2+}]$ changes, a set of EGTA experiments were performed. In these experiments, 1 mM EGTA and 3 mM DM-nitrophen were added to the *cis* solution. The resting free $[Ca^{2+}]$ was carefully titrated to pCa 7. The Ca^{2+} liberated by a flash was then chelated by the EGTA (Fig. 2 *D*). The fast rise in free $[Ca^{2+}]$ was due to liberation from DM-nitrophen. The fall in free $[Ca^{2+}]$ was due to chelation by EGTA. Thus, the local electrode can track slow $[Ca^{2+}]$ changes.

After a flash, the photolyzed solution directly in front of the bilayer was exchanged with unphotolyzed solution by stirring the bath. The time course of this exchange was followed by the local Ca^{2+} electrode as illustrated in Fig. 2 *E*. A flash was applied while the bath was being continuously stirred. The local free $[Ca^{2+}]$ rapidly rose, then slowly returned to baseline (pCa 7). At least 30 s of stirring was required to ensure adequate washout of photolyzed solution after a flash.

The reason for developing this photolysis apparatus was to define the fast kinetics of Ca^{2+} regulation of single SR Ca^{2+} release channels. Channels were reconstituted and sidedness was determined by ATP sensitivity (Fill et al., 1990). To maintain strict control of the $[Ca^{2+}]$ near the channel, Cs^+ was used as charge carrier. The identity of the channel was confirmed by the pharmacological probe ryanodine. The action of 10 μM ryanodine on the cardiac Ca^{2+} release channel is illustrated in Fig. 3 *A*. Ryanodine induced the characteristic changes in channel behavior, slow gating to a subconduction level (Rousseau et al., 1989). Ryanodine had the same action on the skeletal Ca^{2+} release channel (not shown).

The steady-state Ca^{2+} activation of both cardiac and skeletal channels is summarized in Fig. 3 *B*. These experiments were performed by measuring open probability (P_o) over several minutes at different steady-state free $[Ca^{2+}]$s. The free $[Ca^{2+}]$ was adjusted using the bulk Ca^{2+} electrode. The mean P_o at pCa 5 were 0.63 ± 0.06 (SE; n = 6) and 0.21 ± 0.07 (n = 8) for the cardiac and skeletal channels, respectively. The steady-state Ca^{2+} dependencies of cardiac (*filled circles*) and skeletal channels (*open circles*) are plotted in Fig. 3 *B*. The data were normalized to the mean P_o at pCa 5. Normalized P_o was plotted against the myoplasmic (*cis*) free $[Ca^{2+}]$. The slopes ($n_H \approx 1.0$) and the apparent K_D values ($\approx 2 \times 10^{-6}$ M) were nearly identical for both cardiac and skeletal Ca^{2+} release channels.

Steady-state experiments, however, provide no information concerning the rate at which these channels respond to changes in $[Ca^{2+}]$. To determine how quickly Ca^{2+} activates these channels, fast free $[Ca^{2+}]$ stimuli were applied by flash photolysis of DM-nitrophen. The first steps in the flash photolysis experiment are illustrated in Fig. 4. Single channels were reconstituted at the optimal steady-state free $[Ca^{2+}]$ (standard solution: pCa 5). In the standard solution, channel number and bilayer viability were determined (Fig. 4 *A*). Channel number was estimated by observing channel activity over many minutes (Colquhoun and Howkins, 1983). Bilayer viability was inversely proportional to the baseline leak current. Experiments were done only on bilayers that had a small leak current and contained one channel. DM-nitrophen (3 mM) was then added to the *cis* chamber (cytoplasmic side of channel). The free $[Ca^{2+}]$ was carefully titrated to pCa 7 using the bulk Ca^{2+} electrode. In the presence of DM-nitrophen (pCa 7), the P_o was essentially zero (Fig. 3 *B*).

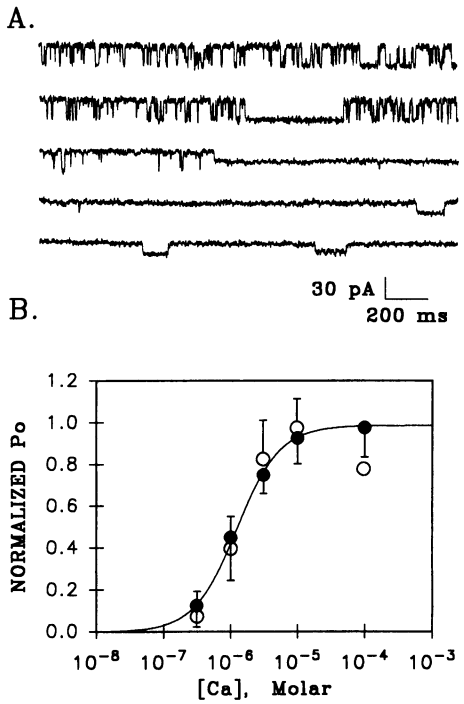

FIGURE 3 Steady-state Ca^{2+} activation of the cardiac and skeletal SR Ca^{2+} release channels. (*A*) Sequential single channel records illustrating the action of ryanodine (10 μM) in the presence of 10 μM free Ca^{2+}. Single channel open events are shown as upward deflections. Ryanodine "locks" the channel in a slow gating, subconductance state. Records shown were from a cardiac channel but are representative of ryanodine action on both channel isotypes. (*B*) The relationship between open probability (P_o) and free [Ca^{2+}]. Data are reported as mean ± SE for both cardiac (●) and skeletal (○) channels. Data points were fit with the Hill equation ($n_H = 1$; $K_D \approx 2 \times 10^{-6}$).

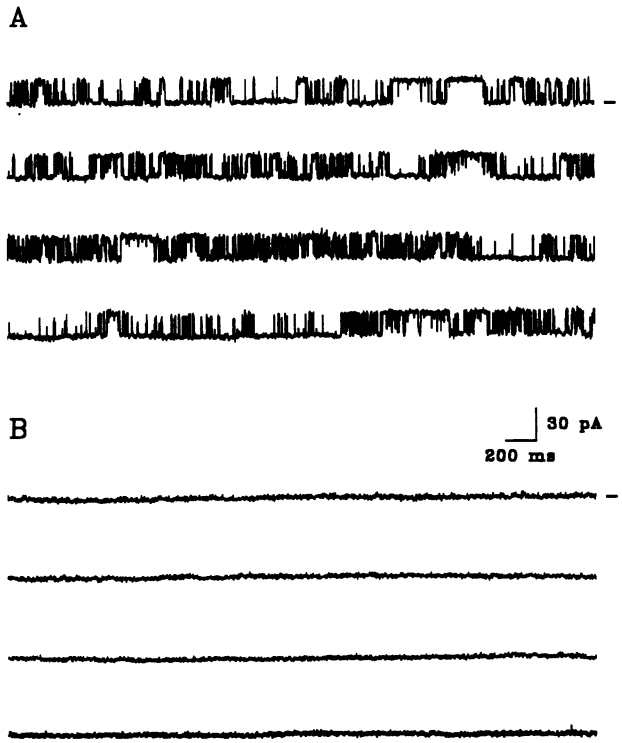

FIGURE 4 Initial steps in a flash photolysis experiment. Records shown are from a cardiac channel but are also representative of a skeletal channel. Single channel open events are shown as upward deflections. (*A*) Typical records immediately after incorporation of a channel into bilayer. Solution contains 10 μM free Ca^{2+}. (*B*) Typical records after addition of DM-nitrophen (3 mM; free [Ca^{2+}] titrated to 100 nM).

Many identical precalibrated, photolytically induced, local free [Ca^{2+}] steps (pCa 7→6) were applied to both cardiac and skeletal channels (Fig. 5). After each UV flash, resting conditions (pCa 7) were reestablished by stirring the bath. Stirring was stopped, for at least 15 s, before the next flash to allow time for solution movement to stop. The average interval between flashes varied from 103 to 130 s. Up to 150 flashes could be applied to the same channel using this flash: stir:flash protocol. The bath free [Ca^{2+}] was continuously monitored and when necessary re-adjusted to pCa 7.

In Fig. 5, representative single channel records from cardiac and skeletal release channels are shown. In each group (cardiac or skeletal), records were aligned with the flash (*arrow* at bottom). Before the flash, the free [Ca^{2+}] was pCa 7 and the single-channel P_o was near zero. At the flash, free [Ca^{2+}] rapidly increased to pCa 6 and remained constant at pCa 6 throughout the rest of the sweep (see Fig.2 *B*). At the flash, single channel P_o increased rapidly to near unity and then slowly decayed.

To quantitate the time course of channel activation at least 50 single-channel records were combined to generate ensemble averages. The ensemble averages consisted of sets of identical sweeps on the same channel. The channel was repetitively activated with a standard stimuli, taking care to wait between stimuli so that the channel was always in same distribution of resting states at pCa 7. Thus, the ensemble averages represented the channel open probability as a function of time. To simplify analysis and interpretation, experiments were only performed on bilayers containing one channel. Analysis of the ensemble averages was based on the arguments of Aldrich and Yellen (1983) for analysis of the nonstationary channel kinetics of voltage-dependent channels.

Channel open probability increased exponentially after photolysis of DM-nitrophen. The time constant of this response was determined from the fit of a single exponential curve. This time constant was reported as the activation rate of the channel. The stimuli and the temporal resolution of our single channel records were at least 10 times faster than the measured activation rate. Thus, the rise time of the ensemble average was assumed to be a good representation of the single channel activation rate.

In Fig. 6, representative ensemble currents (*top*) and their associated fits (*bottom*) are shown for both the cardiac and skeletal release channels. The data points (*below*) are subsets of the data points plotted (*above*). The time constants of activation for the cardiac and skeletal channels were 1.43 ± 0.65 (mean ± SD; $n = 5$) and 1.28 ± 0.61 ($n = 5$), respectively. There was no significant ($p > 0.001$; unpaired two-tailed t-test) difference between the rate that Ca^{2+} activates the cardiac and skeletal isoforms of the channel.

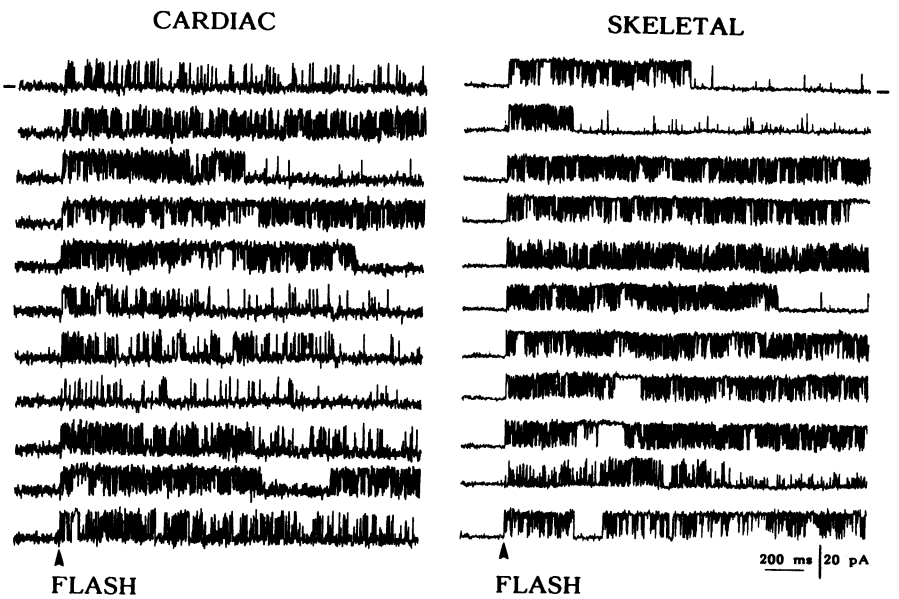

FIGURE 5 Activation of cardiac and skeletal Ca^{2+} release channels by photolysis of caged Ca^{2+}. In the presence of DM-nitrophen (3 mM), more than 50 UV flashes were applied to each channel. Eleven representative sweeps from a cardiac and a skeletal SR Ca^{2+} release channel experiment are shown. Single channel openings are shown as upward deflections. Identical UV laser flashes were applied at the arrow (*bottom*). The initial (*before arrow*) free [Ca^{2+}] was 100 nM. The laser flash elevated the free [Ca^{2+}] to 1 μM. Between UV flashes, the initial conditions were reestablished by stirring the bath.

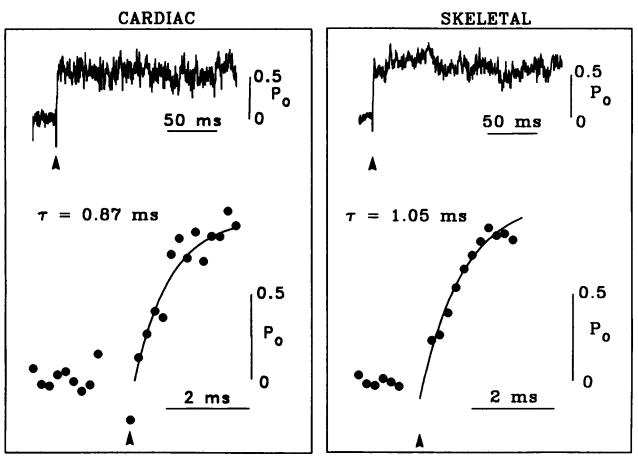

FIGURE 6 Time course of activation of single cardiac and skeletal muscle Ca^{2+} release channels by photolysis of caged Ca^{2+}. Representative ensemble currents (*top* traces) are shown. Ensemble currents were generated from addition of 56 (cardiac) and 79 (skeletal) single channel sweeps. The time courses of activation were best fit by a single exponential (*lower* traces, expanded scale). For the records shown, the time constants are 0.87 and 1.05 ms for the cardiac and skeletal, respectively. Data are representative of five cardiac and five skeletal channel experiments.

The steady-state Ca^{2+}-dependence (Fig. 3 *B*) indicates that at pCa 6 the P_o should be relatively low and constant. However, the photolytic stimuli (pCa 7→pCa 6) consistently and almost maximally activated both the cardiac and skeletal channels. The P_o shortly after the flash was near unity. This discrepancy between the steady-state Ca^{2+} dependence ([Ca^{2+}] versus P_o; Fig. 3 *B*) and the Ca^{2+} dependence immediately after the photolytic stimuli might be explained by single-channel adaptation where the channel's sensitivity decreases during sustained exposure to a higher Ca^{2+} level (Györke and Fill, 1993).

Adaptation was defined in cardiac RyR channels (Györke and Fill, 1993). In the cardiac channel, a Ca^{2+} sensitivity shift results in a spontaneous decay in P_o after the photolytic stimuli. To ascertain whether skeletal RyR channels respond in a similar fashion, single-channel activity was recorded for an extended period after the standard photolytic stimuli. Fig. 7 shows an ensemble average generated from 67 single-channel sweeps. Note the expanded time scale. The P_o decays spontaneously to a low value after the flash. In Fig. 7 *B*, single exponential curves were fit to both the activation and the spontaneous decay. The spontaneous decay had a time constant of ≈3.4 s. Thus, the skeletal and cardiac channels respond to the standard stimuli (pCa 7→pCa 6) similarly. This is consistent with the possibility that adaptation is a property of single skeletal RyR channels.

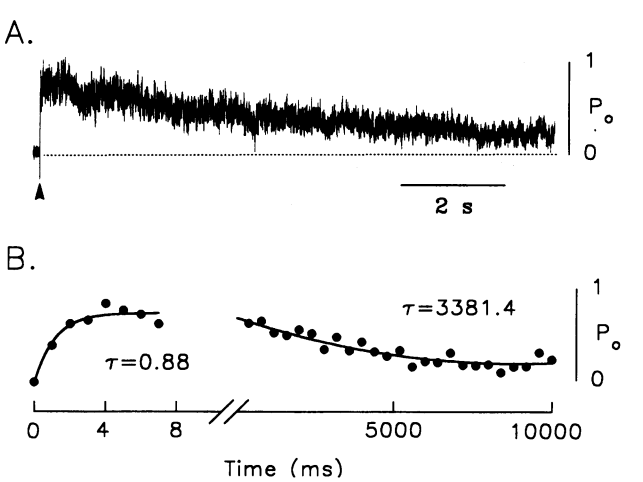

FIGURE 7 Single skeletal RyR/Ca^{2+} release channel P_o measured for an extended period after the standard photolytic Ca^{2+} stimulus. Ensemble current was generated from 67 single channel sweeps. The flash was applied at the arrow. Between UV flashes, the initial conditions were reestablished by stirring the bath. Data are representative of four experiments.

DISCUSSION

In this paper, flash photolysis of caged-Ca^{2+} was used to define the Ca^{2+} activation time course of individual SR Ca^{2+} release channels incorporated in planar lipid bilayers. The same flash photolysis technique was previously used to describe a novel type of Ca^{2+} regulation in cardiac release channels, single-channel adaptation (Györke and Fill, 1993). This paper details the method and compares the rate of Ca^{2+} activation in cardiac and skeletal channels. The essence of the method is the delivery of laser light pulses through a single optic fiber micropositioned directly in front of the bilayer. These UV light pulses liberate Ca^{2+} from DM-nitrophen, a photolabile Ca^{2+} chelator. Because photolysis occurs only in a small volume near the bilayer, photolyzed solution was replaced with unphotolyzed solution by simply stirring the bath. A flash:stir:flash experimental protocol allows photolytic Ca^{2+} stimuli to be applied repetitively to the same channel. This is essential for obtaining the ensemble averages required to quantitate the nonstationary kinetics of the channel's response.

One difficulty in any flash photolysis method is the inherent uncertainty about the efficiency of photolysis. To measure directly the $[Ca^{2+}]$ changes induced by photolysis in our bilayer experiments, the bilayer aperture itself was transformed into a Ca^{2+}-sensitive electrode. The same flash protocol applied in the experiment was then applied to this Ca^{2+} electrode. This Ca^{2+} electrode method has several advantages. The geometric relationship between the bilayer aperture and the micropositioned optic fiber is unchanged. It allows experimentation and calibration to be done in the same solution. The primary disadvantage of the method is its relatively poor time resolution (response time: 50–100 ms). Although use of a fluorescent Ca^{2+} indicator would improve time resolution, certain technical considerations make an optical approach impractical at this time.

In our application, photolysis-induced $[Ca^{2+}]$ changes were actually measured at the point of action (i.e., in the plane of the bilayer) by the local Ca^{2+} electrode described above. If photolysis efficiency decays along the light path (optic fiber→bilayer), then the $[Ca^{2+}]$ will vary along this path. It is possible that Ca^{2+} diffusion along the light path influences the measured channel kinetics. If Ca^{2+} diffusion influences the kinetics, then the Ca^{2+} activation rate would vary as a function of light path length. In control experiments where the optic fiber was micropositioned different distances from the bilayer, the Ca^{2+} activation rate was not significantly different. Thus, diffusion does not appear to dominate the measured kinetics. In any event, there would be no impact on the cardiac/skeletal Ca^{2+} activation rate comparison because identical photolytic stimuli were applied to both channels.

Upon photolysis, the Ca^{2+} affinity of DM-nitrophen changes from $\approx 10^{-9}$ to $\approx 10^{-3}$ M (Kaplan and Ellis-Davies, 1988). It has been suggested that under certain conditions Ca^{2+} liberated rapidly from the photolyzed DM-nitrophen re-equilibrates with unphotolyzed free DM-nitrophen with a rate constant of ≈ 1 ms (Zucker, 1993). The result would be a large fast $[Ca^{2+}]$ spike that would not be resolved by our Ca^{2+} electrode method. To minimize the possible $[Ca^{2+}]$ spikes, the resting free $[Ca^{2+}]$ of our DM-nitrophen solutions was carefully adjusted to 10^{-7} M so that little unphotolyzed DM-nitrophen existed. Because the K_D of DM-nitrophen is 10^{-9}, the DM-nitrophen essentially would be saturated (99%) with Ca^{2+}. If the total [DM-nitrophen] was 3 mM, then about 30 μM free DM-nitrophen would be present. Vergara and Escobar (1993) have argued that $[Ca^{2+}]$ spikes may not occur with DM-nitrophen buffering Ca^{2+} at 10^{-7} M. In their experiments, a sufficiently fast fluorescent Ca^{2+} indicator (Ca^{2+} green-5N; Molecular Probes, Eugene, OR) with an appropriate K_D was used. We have demonstrated that if the Ca^{2+} spikes do occur that they do not activate the cardiac RyR under the experimental conditions used here (Lamb et al., 1994). If fast $[Ca^{2+}]$ spikes occur, it would not change our data interpretation because identical Ca^{2+} stimuli were applied to both channel isoforms.

The steady-state Ca^{2+} dependencies of single cardiac and skeletal RyR channels were indistinguishable. Spontaneous channel activity increased as the steady-state free $[Ca^{2+}]$ was raised from 100 nM to 100 μM. At 1 μM steady-state free Ca^{2+}, single-channel P_o was significantly less than unity. However, when the free $[Ca^{2+}]$ was rapidly changed to 1 μM by photolysis, P_o immediately after the flash was near unity. This implies that the Ca^{2+} sensitivity of the channel immediately after the fast $[Ca^{2+}]$ step was different (greater) than the Ca^{2+} sensitivity under steady-state conditions. A similar observation was explained by a gradual shift in Ca^{2+} sensitivity that was proposed to underlie single-channel adaptation (Györke and Fill, 1993). In cardiac RyR channels, the Ca^{2+} sensitivity shift mediates a spontaneous decay in activity after a photolytic $[Ca^{2+}]$ step. In this study, we show that the skeletal RyR channel activity also spontaneously decays after a sustained stimuli (Fig. 7). This suggests that adaptation is also a property of the skeletal RyR channel. Alternatively, such properties could arise if the photolysis of DM-nitrophen were not homogeneous. It is possible that "Ca^{2+} hot spots" (i.e., areas of high $[Ca^{2+}]$) occur. The phenomena could occur if the channel activates in response to a local "Ca^{2+} hot spot." Local "hot spots" would be undetectable by our Ca^{2+} electrode method.

The fast kinetics of SR Ca^{2+} release measured with millisecond resolution have been defined previously in SR vesicle populations in both skeletal (Meissner et al., 1986) and cardiac (Meissner and Henderson, 1987) muscle. These vesicle studies demonstrate that the rate of SR Ca^{2+} release is adequate to explain CICR in situ. In our study, we show that the Ca^{2+} activation rates of single cardiac and skeletal channels are nearly identical. The Ca^{2+} activation rates we measured with microsecond resolution are roughly 10-fold faster than the rates of SR Ca^{2+} release measured in SR vesicle preparations.

Precisely defining the rate of Ca^{2+} activation of cardiac and skeletal ryanodine-sensitive SR Ca^{2+} release channels is important to understanding E-C coupling. For many years,

Ca^{2+} was considered to be the trigger of SR Ca^{2+} release in both skeletal (Ford and Podolsky, 1970; Frank, 1980) and cardiac muscle (Fabiato, 1983). It is now generally accepted that CICR is the mechanism that underlies E-C coupling in cardiac muscle (Stern and Lakata, 1992). In skeletal muscle, E-C coupling appears to be mediated through a direct communication between the ryanodine receptor channel and the dihydropyridine receptor (DHPR), a T-tubule protein (Rios and Pizarro, 1991; Rios et al., 1991)

The cardiac and skeletal SR Ca^{2+} release ryanodine receptor channels have many similarities. They share significant amino acid homology (Nakai et al., 1990; Takeshima et al., 1989). They operate in comparable morphological environments (Franzini-Armstrong and Nunzi, 1983; Sommer and Johnson, 1979). The steady-state Ca^{2+} dependencies of isolated single cardiac and skeletal SR Ca^{2+} release channel are nearly identical (Fig. 3 *B*). Further, site-directed mutagenesis of another protein, the DHPR, changes E-C coupling from skeletal- to cardiac-like (Tanabe et al., 1990). Thus, skeletal and cardiac E-C coupling differences do not appear to be due to ryanodine receptor subtype. The only obvious difference between the two ryanodine receptor subtypes appears to be a direct functional link to the DHPR.

Morphological evidence indicates that half of the SR Ca^{2+} release channels in skeletal muscle are not associated with DHPRs (Franzini-Armstrong and Nunzi, 1983). These channels, therefore, could be regulated in a cardiac-like (CICR) fashion (Rios and Pizarro, 1991). In skeletal muscle fibers, this possibility has been tested by attempts to block CICR either by placing a high affinity Ca^{2+} buffer in the myoplasm (Jacquemond et al., 1991) or by applying certain local anesthetics (Pizarro et al., 1989). The results show that these perturbations inhibit a fast transient component of the global intracellular Ca^{2+} release signal leaving a smaller long-lived component. This suggests that the fast transient component is due to CICR. We have demonstrated that the Ca^{2+} activation rate of single isolated ryanodine-sensitive skeletal SR Ca^{2+} release channels is adequately fast to mediate that fast transient component.

We show that the Ca^{2+} sensitivity and Ca^{2+} activation rate of single cardiac and skeletal SR Ca^{2+} release channels are similar when reconstituted in an artificial planar bilayer. In the cell, however, there may be other factors that regulate CICR. It has been suggested that the Ca^{2+} activation site on the channel may be morphologically restricted in situ (Györke and Palade, 1993). This could explain why isolated channels might respond to a Ca^{2+} stimuli, whereas the same channels in situ do not. Alternatively, physiologically important ligands, which were absent in our study, may modulate the Ca^{2+} regulation of the channel. For example, skeletal SR Ca^{2+} release channels are more sensitive to Mg^{2+} than cardiac channels (Meissner and Henderson, 1987). At physiological Mg^{2+} levels, it is possible that CICR in skeletal muscle is inhibited.

Supported by NIH AR41197 (M. Fill), M. D. A. (S. Györke), and Fondecyt 1294–91 (B. Suárez-Isla).

REFERENCES

Aldrich R. W., and G. Yellen. 1983. Analysis of nonstationary channel kinetics. *In* Single-Channel Recording. B. Sakmann and E. Neher, editors. Plenum Press, New York. 287–299.

Armstrong, C. M., F. Bezanilla, and P. Horowicz. 1972. Twitches in the presence of EGTA. *Biohim. Biophys. Acta.* 267:605–608.

Chu, A., M. Fill, E. Stefani, and M. L. Entman. 1993. Cytoplasmic Ca^{2+} does not inhibit the cardiac muscle sarcoplasmic reticulum ryanodine receptor Ca^{2+} channel, although Ca^{2+}-induced Ca^{2+} inactivation of Ca^{2+} release is observed in native membranes. *J. Membr. Biol.* 135:49–59.

Cleemann, L., and M. Morad. 1991. Role of Ca^{2+} channel in cardiac excitation-contraction coupling in the rat: evidence from Ca^{2+} transients and contraction. *J. Physiol. (Lond.).* 432:283–312.

Colquhoun, D., and A. G. Howkins. 1983. The principles of stochastic interpretation of ion-channel mechanisms. *In* Single-Channel Recording. B. Sakmann and E. Neher, editors. Plenum Press, New York. 135–174.

Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* 57:71–108.

Fabiato, A. 1983. Calcium induced release of calcium from the cardiac sarcoplasmic reticulum. *Am. J. Physiol.* 245:C1–C14.

Fabiato, A. 1985. Time and calcium dependence of activation and inactivation of calcium-induced release of calcium from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J. Gen. Physiol.* 85: 247–289.

Fill, M., R. Coronado, J. R. Mickelson, J. Vilven, J. Ma, B. A. Jacobson, and C. F. Louis. 1990. Abnormal ryanodine receptor channels in malignant hyperthermia. *Biophys. J.* 57:471–475.

Ford, L. E, and R. J. Podolsky. 1970. Regenerative calcium release within muscle cells. *Science.* 167:58–59.

Frank, G. B. 1980. The current view of the source of trigger calcium in excitation-contraction coupling in vertebrate skeletal muscle. *Biochem. Pharmacol.* 29:2399–2406.

Franzini-Armstrong, C., and G. Nunzi. 1983. Junctional feet and particles in the triads of a fast twitch muscle fibre. *J. Muscle Res. Cell Motil.* 4:233–252.

Györke, S., and M. Fill. 1993. Ryanodine receptor adaptation: control mechanism of Ca^{2+}-induced Ca^{2+} release in heart. *Science.* 260:807–809.

Györke, S., and P. Palade. 1993. Role of local Ca^{2+} domains in activation of Ca^{2+}-induced Ca^{2+} release in crayfish muscle fibers. *Am. J. Physiol.* 264:C1505–C1512.

Jacquemond, V., L. Csernoch, M. G. Klein, and M. F. Schneider. 1991. Voltage-gated and calcium-gated calcium release during depolarization of skeletal muscle fibers. *Biophys. J.* 60:867–873.

Kaplan, J. H., and G. C. R. Ellis-Davies. 1988. Photolabile chelators for the rapid photorelease of divalent cations. *Proc. Natl. Acad. Sci. USA.* 85: 6571–6575.

Lai, F. A., H. P. Erickson, E. Rousseau, Q-Y. Liu, and G. Meissner. 1988. Evidence for a Ca^{2+} channel within the ryanodine receptor complex from cardiac sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 151: 441–449.

Lamb, G. D., M. W. Fryer, D. G. Stephenson, S. Gyorke, and M. Fill. 1994. Technical Comment: Ca^{2+}-induced Ca^{2+} release in response to flash photolysis. *Science.* 263:986–988.

McCray, J. A., N. Fidler, G. Lim, C. R. Ellis-Davies, and J. H. Kaplan. 1992. Rate of release of Ca^{2+} following laser photolysis of the DM-nitrophen-Ca^{2+} complex. *Biochemistry.* 31:8856–8861.

Meissner, G., E. Darling, and J. Eveleth. 1986. Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. *Biochemistry.* 25:236–244.

Meissner, G., and J. S. Henderson. 1987. Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotide and calmodulin. *J. Biol. Chem.* 262:3065–3072.

Nakai, J., T. Imagawa, Y. Hakamat, M. Shigekawa, H. Takeshima, and S. Numa. 1990. Primary structure and functional expression from cDNA of the cardiac ryanodine receptor/calciun release channel. *FEBS Lett.* 271: 169–177.

Pizarro, G. L., L. Chernoch, I. Uribe, and E. Rios. 1989. Tetracaine and pathways of Ca^{2+} release in skeletal muscle. *Biophys. J.* 55:237*a*. (Abstr.)

Rios, E., J. Ma, and A. Gonzalez. 1991. The mechanical hypothesis of

excitation-contraction (EC) coupling in skeletal muscle. *J. Muscle Res. Cell Motil.* 12:127–135.

Rios, E., and G. Pizarro. 1991. Voltage sensor of excitation-contraction coupling in skeletal muscle. *Physiol. Rev.* 71:849–908.

Rousseau, E., and G. Meissner. 1989. Single cardiac sarcoplasmic reticulum Ca^{2+}-release channel: activation by caffeine. *Am. J. Physiol.* 256: H328–H333.

Rousseau, E., J. S. Smith, and G. Meissner. 1989. Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channel. *Am. J. Physiol.* 253:C364–C368.

Sipido, K., and W. G. Wier. 1991. Flux of calcium across the sarcoplasmic reticulum of guinea-pig cardiac cells during excitation-contraction coupling. *J. Physiol.* 435:605–630.

Smith, J. S., R. Coronado, and G. Meissner. 1986. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. *J. Gen. Physiol.* 88:573–588.

Smith, J. S., T. Imagawa, J. Ma, M. Foll, K. P. Campbell, and R. Coronado. 1988. Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *J. Gen. Physiol.* 92: 1–26.

Sommer, J. R., and E. A. Johnson. 1979. Ultrastructure of cardiac muscle. *In* Handbook of Physiology. Vol. 1, Section 2. The Cardiovascular System. R. M. Berne, editor. American Physiological Society, Bethesda, MD. 113–186.

Spiecker, W., and H. C. Luttgau. 1979. Extracellular calcium and excitation-contraction coupling. *Nature.* 280:158–160.

Stern, M. D., and E. G. Lakatta. 1992. Excitation-contraction coupling in the heart: the state of the question. *FASEB J.* 6:3092–3100.

Takeshima, H., S. Nishumara, T. Matsumoto, H. Ishida, K. Kangawa, N. Minamino, H. Matsuo, M. Ueda, M. Hanaoka, T. Hirose, and S. Numa. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature.* 339:439–445.

Tanabe, T., A. Mikami, S. Numa, and K. Beam. 1990. Cardiac-type excitation-contraction coupling in disgenic skeletal muscle injected with cardiac dihydroyridine receptor cDNA. *Nature.* 344:451–453.

Tate, C. A., R. J. Bick, A. Chu, W. B. Van Winkle, and M. L. Entman. 1985. Nucleotide specificity of canine cardiac sarcoplasmic reticulum. GTP-induced calcium accumulation and GTPase activity. *J. Biol. Chem.* 260: 9618–9623.

Vergara, J., and M. Delay. 1986. A transmission delay and the effect of temperature at the triadic junction of skeletal muscle. *Proc. R. Soc. Lond. B Biol. Sci.* 229:97–110.

Vergara, J., and A. Escobar. 1993. Detection of Ca^{2+} transients in skeletal muscle fibers using the low affinity dye calcium-green-5N. *Biophys. J.* 65:37*a*. (Abstr.)

Zucker, R. S. 1993. The calcium concentration clamp: spikes and reversible pulses using the photolabile chelator DM-nitrophen. *Cell Calcium.* 14: 87–100.