Adaptation of Single Cardiac Ryanodine Receptor Channels

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ABSTRACT Single cardiac ryanodine receptor (RyR) channel adaptation was previously defined with Ca^{2+} stimuli produced by flash photolysis of DM-nitrophen (caged-Ca^{+2}). Photolysis of DM-nitrophen induced a very fast Ca^{+2} overshoot (Ca^{+2} spike) at the leading edge of the Ca^{+2} stimuli. It has been suggested that adaptation ($\tau \approx 1.3$ s) may reflect Ca^{+2} slowly coming off the RyR Ca^{+2} activation sites following the faster Ca^{+2} spike ($\tau \approx 1$ ms). This concern was addressed by defining the Ca^{2+} deactivation kinetics of single RyR channels in response to a rapid reduction in free Ca^{2+} concentration ($[Ca^{2+}]_{FREE}$). The $[Ca^{2+}]_{FREE}$ was lowered by photolysis of Diazo-2. Single RyR channels deactivated ($\tau \approx 5.3$ ms) quickly in response to the photolytically induced $[Ca^{2+}]_{FREE}$ reduction. Improved estimates of the Ca^{2+} spike time course indicate that the Ca^{2+} spike is considerably faster (10–100-fold) than previously thought. Our data suggest that single RyRs are not significantly activated by fast Ca^{2+} spikes and that RyR adaptation is not due to deactivation following the fast Ca^{2+} spike. Thus, RyR adaptation may have an important impact on Ca^{2+} signaling in heart.

INTRODUCTION

The ryanodine receptor (RyR) channel mediates the process of Ca^{+2}-induced Ca^{+2} release in heart. The mechanism of Ca^{+2}-induced Ca^{+2} release, an inherently self-regenerating process, is precisely controlled in vivo (Bers, 1991). It is generally believed that a negative feedback mechanism must exist to counter the inherent positive feedback of Ca^{+2}-induced Ca^{+2} release. Fabiato (1985) proposed that the negative feedback mechanism may be Ca^{+2}-dependent inactivation. However, studies of patch-clamped myocytes and single cardiac RyR channels have not conclusively confirmed the existence of Ca^{+2}-dependent inactivation (Cleemann and Morad, 1991; Nabauer and Morad, 1990; Chu et al., 1993; Laver et al., 1995). Recently, Györke and Fill (1993) proposed that the negative feedback mechanism may be adaptation instead of inactivation.

The adaptation proposal is based on the demonstration that single RyR channels respond transiently when the $[Ca^{2+}]_{FREE}$ is elevated rapidly (Györke and Fill, 1993; Valdivia et al., 1995). Open probability (P_o) peaked and then spontaneously decayed in the continued presence of elevated Ca^{2+}. The spontaneous decay was not due to Ca^{2+}-dependent inactivation because a second Ca^{2+} elevation activated the apparently inactivated channels (Györke and Fill, 1993). At the whole-cell level, Ca^{2+} release phenomena consistent with the existence of RyR adaptation have been observed (Rios, 1994; Yasui et al., 1994; Györke and Györke, 1996). Adapting RyR channels would explain why fast Ca^{2+} stimuli are more effective at activating Ca^{2+} release than are slow Ca^{2+} stimuli (Fabiato, 1985). Adapting RyR channels would explain why apparently inactivated RyR channels in cells are reactivated by tail Ca^{2+} currents (Yasui et al., 1994, Cleeman and Morad, 1991). Adapting RyR channels would also explain why single RyR channels in bilayers do not become refractory at relatively high cytoplasmic $[Ca^{2+}]$ values (0.1–1.0 mM; Chu et al., 1993). Thus, the possibility that single RyR channels adapt would reconcile a large body of apparently contradictory results collected over the past decade.

Györke and Fill (1993) defined adaptation with Ca^{2+} stimuli produced by flash photolysis of caged Ca^{2}. The caged-Ca^{2+} compound used, DM-nitrophen, is a photolabile high-affinity Ca^{+2} buffer (Kaplan and Ellis-Davies, 1988). Photolysis of DM-nitrophen, however, can produce a large fast $[Ca^{2+}]_{FREE}$ overshoot on the leading edge of the sustained $[Ca^{2+}]_{FREE}$ elevation (McCray et al., 1992; Zucker, 1993; Escobar et al., 1995; Ellis-Davies et al., 1996). The overshoot (i.e., the Ca^{2+} spike) occurs because photolysis liberates Ca^{+2} from the DM-nitrophen–Ca^{2+} complex faster than free DM-nitrophen binds Ca^{2+}. It has been suggested that RyR adaptation ($\tau \approx 1.3$ s; Györke and Fill, 1993; Valdivia et al., 1995) could be driven by the Ca^{+2} spike if RyR Ca^{+2} deactivation were slow enough (i.e., the Ca^{2+} spike activates RyR; then activity decays as Ca^{2+} slowly comes off the Ca^{+2} activation site(s) following the fast Ca^{+2} spike; Lamb and Stephenson, 1995).

To predict the effect of the Ca^{+2} spike on RyR activation and adaptation requires defining the kinetics of the Ca^{+2} spike and the RyR channel. Recently, measurements with improved temporal resolution have revealed that the Ca^{2+} spike is much faster than previously thought (Escobar et al., 1995). Here we have used model simulations of the Ca^{2+} spike based on Escobar et al. (1995) to estimate better the Ca^{2+} spike kinetics. Experimentally, the deactivation kinetics of single RyR channels to a fast $[Ca^{2+}]$ reduction were

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defined. Single RyR channels activate ($\tau \approx 1$ ms) and deactivate ($\tau \approx 5.3$ ms) quickly in response to fast $[Ca^{2+}]_{FREE}$ changes. Thus, the RyR channel has relatively fast on/off kinetics, which should allow it to track (react to) $[Ca^{2+}]_{FREE}$ signals that last only a few milliseconds. Our data suggest that single RyRs in bilayers are not significantly activated by the fast Ca^{2+} spike. We also show that adaptation ($\tau \approx 1300$ ms) can not be due to RyR deactivation ($\tau \approx 5.3$ ms) following a Ca^{+2} spike. Instead, it is much more likely that adaptation represents a regulatory mechanism that may be important in the control of intracellular Ca^{2+} signaling in heart.

MATERIALS AND METHODS

Sarcoplasmic reticulum microsome preparation

Heavy sarcoplasmic reticulum microsomes were prepared from dog cardiac muscle as previously described (Tate et al., 1985). Briefly, canine left ventricle was diced and homogenized. Differential centrifugation was used to isolate sarcoplasmic reticulum microsomes. The microsomes were stored in 0.3 M sucrose, 0.9% NaCl, 10 mM Tris maleate, pH 6.8, at −80°C until used.

Channel incorporation and single-channel recording

Planar lipid bilayers were formed across a 200-μm-diameter aperture in the wall of a Delrin (Small Parts, Inc., Miami Lakes, FL) cup. Lipid bilayer-forming solution contained an 8:2 (by volume) mixture of phosphatdylethanolamine and phosphatidycholine (Avanti Polar Lipids, Pelham, AL) dissolved in decane at a final concentration of 50 mg/ml. Sarcoplasmic reticulum vesicles were added into one side of the bilayer (defined as *cis*). The other side was defined as *trans* (virtual ground). Standard solutions contained 400 mM $CsCH_3SO_3$ *cis* (20 mM *trans*) and 10 mM HEPES (pH 7.4). The $[Ca^{2+}]_{FREE}$ was measured with a Ca^{+2} electrode. After channel incorporation, the *trans* $CsCH_3SO_3$ was adjusted to 400 mM.

A custom current–voltage-conversion amplifier was used to optimize single-channel recording (Györke and Fill, 1994). Acquisition software (pClamp: Axon Instruments, Foster City, CA), an IBM-compatible 386 computer, and a 12-bit analog/digital–digital/analog converter (Axon Instruments) were used. Single-channel data were digitized at 5–10 kHz and filtered at 1 kHz. Channel sidedness was determined by ATP sensitivity. The orientation of incorporated RyR channels was such that the cytoplasmic side was in the *cis* compartment (Györke and Fill, 1993).

Flash photolysis and Ca^{+2} measurements in the bilayer chamber

Flash photolysis experiments were performed on single RyR channels as previously described (Györke and Fill, 1993; Györke et al., 1994). Briefly, flash photolysis of Diazo-2 (Calbiochem, Inc., San Diego, CA) was used to decrease $[Ca^{+2}]_{FREE}$ rapidly in the microenvironment near single RyR channels. Because binding of Ca^{+2} to Diazo-2 is also very fast (on rate, 8×10^8 M^{-1} s^{-1}; Adams et al., 1989), photolysis of Diazo-2 decreased $[Ca^{+2}]_{FREE}$ in less than 1 ms (Mulligan and Ashley, 1989). Intense 10-ns flashes of UV light were supplied by a frequency tripled, *Q*-switched Nd:YAG laser. Laser light was directed at the bilayer through a 450-μm-diameter fused-silica optic fiber. Only photolabile compound in a small volume of solution between the end of the optic fiber and the bilayer was photolyzed. We could replace photolyzed solution with unphotolyzed solution by simply stirring the bath.

The magnitude of resting $[Ca^{+2}]_{FREE}$ changes was measured as previously described (Györke and Fill, 1993). The local $[Ca^{+2}]$ was monitored by either a Ca^{+2} electrode positioned near the bilayer or a Ca^{+2} electrode formed in the bilayer aperture. Because the response times of the Ca^{+2} electrodes were 30–60 ms, the electrodes were not expected to track very fast changes in $[Ca^{+2}]_{FREE}$. Instead, we used the Ca^{+2} electrodes to determine the steady-state $[Ca^{+2}]_{FREE}$ before and after photolysis.

Measurements and modeling of the fast Ca^{+2} spike

A detailed description of the mathematical model used to estimate the time course of the fast Ca^{2+} spike was presented elsewhere (Escobar et al., 1995). Briefly, the interaction between DM-nitrophen and Ca^{2+} was assumed to be a simple bimolecular process in which the total DM-nitrophen concentration was divided into photolysable and nonphotolysable fractions. The kinetic reaction scheme for both fractions followed a nonstationary simple mass action law. It was assumed that the DM-nitrophen–calcium complex was 2.5 times more sensitive to photolysis than was free DM-nitrophen (Zucker, 1994). To ensure that the large change in DM-nitrophen Ca^{2+} affinity (4.6 nM → 3 mM) on photolysis did not compromise the on-rate constants, kinetic modifications were restricted to the off-rate constants. The numerical integration of the required differential equations was done with the fourth-order Runge–Kutta method of the sCop 3.4 numerical software package.

RESULTS

There is a consensus in the literature that photolysis of DM-nitrophen can produce a fast Ca^{+2} spike at the leading edge of the $[Ca^{+2}]_{FREE}$ change (McCray et al., 1992; Zucker, 1993; Escobar et al., 1995; Ellis-Davies et al., 1996). The Ca^{+2} spike occurs because photolysis of the DM-nitrophen–calcium complex liberates Ca^{+2} faster than free DM-nitrophen binds Ca^{+2}. If there is a mixture of DM-nitrophen–calcium complex and free DM-nitrophen, there will be a Ca^{+2} spike following a flash-photolysis pulse. The estimated kinetics and amplitude of the Ca^{2+} spike will depend on both the experimental conditions and the particular mathematical model used to predict its time course. In the original RyR adaptation study (Györke and Fill, 1993) there was ~140 μM free DM-nitrophen (DM-nitrophen, $K_D \approx 5 \times 10^{-9}$ M; $[Ca^{+2}]_{FREE} = 1 \times 10^{-7}$ M; 2.86 mM DM-nitrophen–calcium complex). Thus, there was a Ca^{+2} spike. Clearly defining the potential effect of that Ca^{2+} spike on a RyR channel is the focus of this paper.

Kinetics of the fast Ca^{+2} spike

The time course of the fast Ca^{+2} spike has been a source of open speculation. Initially, the duration of the Ca^{+2} spike was thought to be several milliseconds (McCray et al., 1992; Zucker, 1993). Recent measurements with improved temporal resolution indicate that the association rate of Ca^{+2} to free DM-nitrophen is much faster than previously thought, i.e., 8×10^7 M^{-1} s^{-1} rather than 1.5×10^6 M^{-1} s^{-1} (Escobar et al., 1995; Zucker, 1993). To calculate the time course of the fast Ca^{+2} spike we applied a model originally described by Escobar et al. (1995). Fig. 1 *A* illustrates Ca^{+2} spike simulations assuming the experimen-

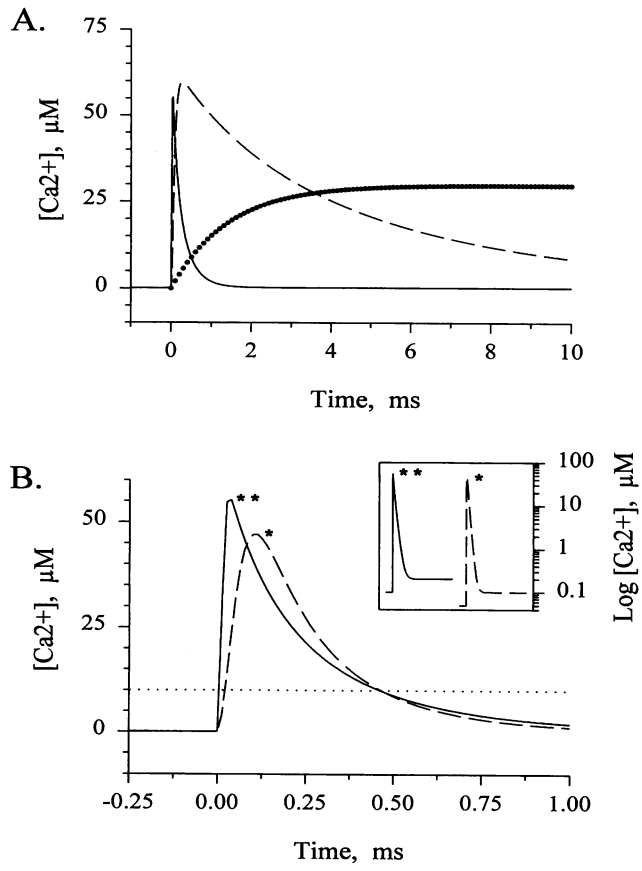

FIGURE 1 Simulated fast Ca^{2+} spikes. A mathematical model was used to estimate the time course of the fast Ca^{2+} spike (Materials and Methods). *A*, Predicted time courses of the fast Ca^{2+} spike assuming the DM-nitrophen Ca^{2+} association rate of Zucher (1993; *dashed curve*) or of Escobar et al. (1995; *solid curve*). The monotonically rising curve reflects the rate of single RyR channel activation. *B*, Simulated Ca^{2+} spikes at resting $[Ca^{2+}]_{FREE}$ of 100 nM (*solid curve*) and 50 nM (*dashed curve*). The same two Ca^{2+} spikes (each trace is 10 ms long) are plotted on a log $[Ca^{2+}]$ scale in the inset. The dotted line represents the $[Ca^{2+}]$ expected to activate RyR channels maximally.

tal conditions used in the initial RyR adaptation study (Györke and Fill, 1993). When the old DM-nitrophen association rate (Zucker, 1993) is used, the Ca^{+2} spike peaks at $\approx$60 μM and decays with a time constant of 8.27 ms. Using the improved estimate of the DM-nitrophen association rate (3×10^7 M^{-1} s^{-1}) the Ca^{+2} spike peaks at $\approx$55 μM and decays much faster ($\tau = 0.55$ ms). The Ca^{+2} spike is at least 15 times faster than previously thought. The time course of RyR channel activation ($\tau \approx 1$ ms) is also shown (*filled circles*). The Ca^{2+} spike simulations shown (Fig. 1) were based on measurements made with the fastest available fluorescent Ca^{2+} indicator (Ca-Orange-5N; Escobar et al., 1995). Unfortunately, the Ca-Orange-5N measurements are still limited by the response time of the indicator. Thus, spike time course estimates may still be temporally limited (i.e., the Ca^{2+} spike could be substantially faster).

Despite the ambiguity concerning the association kinetics of DM-nitrophen, it is clear that the kinetics of the Ca^{+2} spike depend on the free DM-nitrophen concentration. Fig. 1 *B* illustrates simulated Ca^{+2} spikes in the presence of 3 mM total DM-nitrophen at two different values of resting $[Ca^{+2}]_{FREE}$. Because the free DM-nitrophen concentration is inversely proportional to $[Ca^{+2}]_{FREE}$, more free DM-nitrophen was present at the low $[Ca^{+2}]_{FREE}$, and thus the Ca^{+2} spike was faster. At the higher $[Ca^{+2}]_{FREE}$ (low free DM-nitrophen), the Ca^{+2} spike was slower. In the inset of Fig. 1 *B* the same two Ca^{2+} spikes were replotted on a log scale to illustrate better the differences in resting $[Ca^{+2}]_{FREE}$. These particular Ca^{+2} spike simulations (Fig. 1 *B*) were selected because they directly correlate with the Ca^{+2} spikes applied by Györke and Fill (1994). Györke and Fill (1994) showed that the photolytic stimulus producing the Ca^{+2} waveform (labeled * in the figure) did not significantly activate RyR channels, whereas the Ca^{+2} waveform (**) did. Note that values of $[Ca^{2+}]$ above 10 μM (*dotted line*, Fig. 1 *B*) are sufficient for maximum activation of RyR channels (Chu et al., 1993). Consequently, the channels were subjected to maximally activating $[Ca^{2+}]$ for nearly identical periods (456 and 439 μs) in the two cases. Channel activation cannot be easily correlated with changes in Ca^{2+} spike amplitude or duration. A simpler and more logical interpretation is that RyR channel activation was governed by the changes in the steady-state $[Ca^{+2}]_{FREE}$.

Ca^{+2} deactivation of single RyR channels

The response of single RyR channels to a fast $[Ca^{+2}]_{FREE}$ reduction was defined. The fast drop in $[Ca^{+2}]_{FREE}$ was generated by flash photolysis of the caged (Ca^{+2} chelator) Diazo-2 (2 mM). Unfortunately, the Ca^{+2} affinity of Diazo-2 ($K_D \approx 2.2$ μM) limited the $[Ca^{+2}]_{FREE}$ range over which these experiments could be done. In our hands, the maximal Ca^{+2} drop achieved with the commercially available Diazo-2 and a single UV flash was 1×10^{-6} to 0.4×10^{-6} M. Single RyR channels were incorporated into planar lipid bilayers, and the resting $[Ca^{+2}]_{FREE}$ was carefully titrated 1×10^{-6} M by use of a Ca^{+2} electrode. The steady state P_o was monitored, and then single UV flashes were applied to lower the $[Ca^{+2}]_{FREE}$ to 0.4×10^{-6} M. Initial conditions were reestablished between flashes. In Fig. 2 *A* single-channel records illustrate representative channel activity before and after the flash. Data collected from seven single-channel experiments were combined to generate the P_o diary plot shown in Fig. 2 *B*. The P_o was calculated at 5-ms intervals. Only values immediately before and after the flash are shown. The flash occurred 250 ms after the beginning of each data sweep. A single exponential curve was fitted to the P_o diary plot (i.e., the 250–290-ms points). The fit indicates that the P_o decreased with a time constant of 5.29 ms. The average P_o was 0.13 $\pm$ 0.06 (mean $\pm$ SD, $n = 7$) before the flash and 0.03 $\pm$ 0.03 after the flash. Thus, single canine cardiac RyR channels respond rapidly ($\tau \approx 5$ ms) to a sudden decrease in resting $[Ca^{+2}]_{FREE}$.

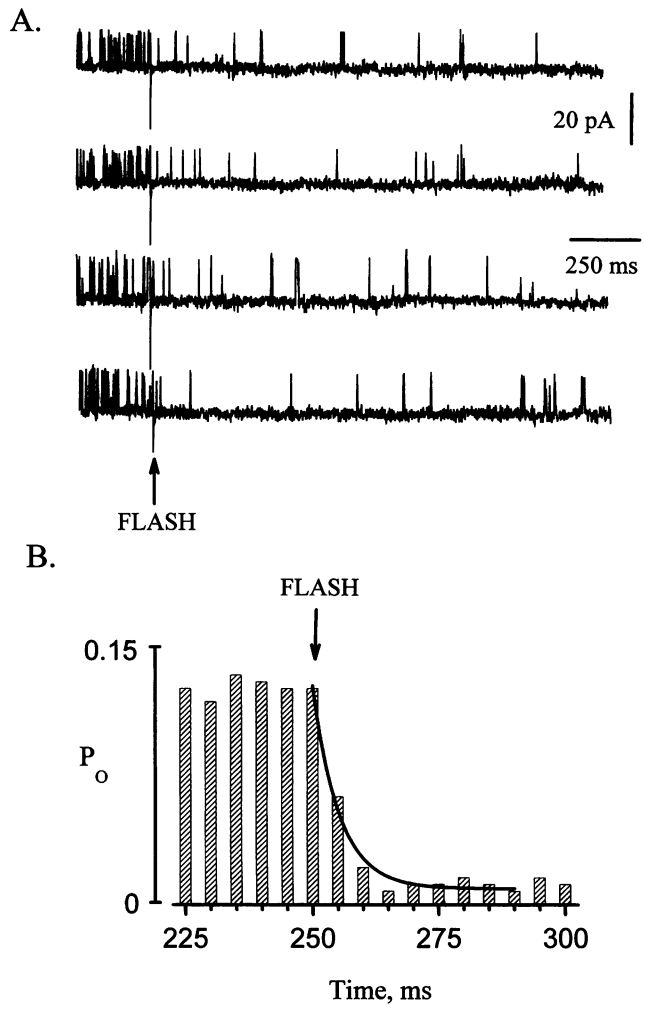

FIGURE 2 Single RyR channel activity rapidly decreases following a fast decrease in the resting $[Ca^{+2}]$. *A*, The four representative single-channel sweeps are from the same single cardiac RyR channel. Open events are current upward deflections (holding potential 30 mV). Sweeps are aligned with the flash (*arrow*). Downward deflection at the arrow in each sweep is a flash artifact. Solutions contained 200 mM $CsCH_3SO_3$, 20 mM Cs HEPES (pH 7.4) and 2 mM Diazo-2 (pCa 6). *B*, The P_o diary plot generated from 7 single-channel experiments (total of 42 single sweeps). The P_o was calculated at 5-ms intervals. The flash occurred at the 250-ms mark (*arrow*). A single exponential (τ = 5.29 ms) was fitted to the data.

DISCUSSION

Do Ca^{2+} spikes alter activation of RyR channels?

Several laboratories have now defined the rate of single RyR channel activation following a fast increase in $[Ca^{+2}]_{FREE}$ (Györke and Fill, 1993; Györke et al., 1994; Valdivia et al., 1995; Sitsapesan et al., 1995; Schiefer et al., 1995). When $[Ca^{+2}]_{FREE}$ was elevated by photolysis of DM-nitrophen, the canine RyR channel activated with a time constant of 1.43 ± 0.65 ms (mean ± SD, n = 5; Györke et al., 1994). When $[Ca^{+2}]_{FREE}$ was elevated by photolysis of NP-EGTA (a related caged-Ca^{+2} compound), the canine RyR channel activated with a time constant of ≈1.35 ms (Valdivia et al., 1995). When $[Ca^{+2}]_{FREE}$ was changed by a relatively slow mechanical method (≈10-ms Ca^{+2} changes), the sheep RyR channel was activated in less than 10 ms (Sitsapesan et al., 1995). When $[Ca^{+2}]_{FREE}$ was changed by a much faster mechanical method, the canine RyR channel was activated in ~2.2 ms (Schiefer et al., 1995). Thus, there is a good consensus that Ca^{2+} activation of single RyR channels is fast, with a time constant of 1–2 ms.

We have shown that the time course of RyR channel activation was monotonic (Györke and Fill, 1993). This is consistent with the activation time course reported by Schiefer et al. (1995). However, the stimulating $[Ca^{+2}]_{FREE}$ waveform in our study contained the fast Ca^{2+} spike, and thus multiple temporal components in the channel activation might be expected. The monotonic nature of RyR activation suggests that either the Ca^{+2} spike did not significantly alter channel activation or the channel was responding maximally, making its activity independent of the $[Ca^{+2}]_{FREE}$ waveform. The latter possibility would be difficult to reconcile with previously published results. Schiefer et al. (1995) showed that the time constant of activation varied (0.2 → 5 ms) with $[Ca^{2+}]$. Györke and Fill (1994) showed that large Ca^{+2} spikes, without superthreshold changes in steady-state $[Ca^{+2}]_{FREE}$ (see the simulations in Fig. 1 *B*), did not saturate the activation process. Györke and Fill (1994) also used photolysis to elevate $[Ca^{2+}]$ from a higher resting Ca^{2+} level (1 μM). In those experiments the Ca^{2+} spike was dramatically attenuated, but RyR channel activation was still fast and monotonic. Thus, the reasonable explanation for the monotonic activation, despite the presence of the Ca^{2+} spike, is that single RyR channels are not significantly activated by the fast Ca^{2+} spike.

Valdivia et al. (1995) also addressed the possibility that the fast Ca^{2+} spike alters RyR channel activation. Valdivia et al. (1995) applied long trains of low-intensity flashes to elevate $[Ca^{+2}]_{FREE}$ progressively. Each flash liberated Ca^{+2} from NP-EGTA and produced a large and relatively slow Ca^{+2} overshoot (Ellis-Davies et al., 1996). Because the free NP-EGTA concentration changed after each flash, the Ca^{+2} spikes varied widely in duration and amplitude. The single RyR channels did not respond to any of the Ca^{+2} spikes. Instead, the RyR channel activated progressively and proportionally to the steady-state $[Ca^{+2}]_{FREE}$ changes. Again, the simplest explanation would be that single RyR channels are not significantly activated by the fast Ca^{+2} spike.

Do Ca^{2+} spikes alter adaptation of RyR channels?

Adaptation was originally proposed as the mechanism that mediates the spontaneous decay in RyR channel activity when the $[Ca^{+2}]_{FREE}$ is elevated from 1×10^{-7} to 1×10^{-6} M by photolysis of DM-nitrophen (Györke and Fill, 1993). Two laboratories have measured the rate of single RyR channel adaptation (Györke and Fill, 1993; Valdivia et

al., 1995). With DM-nitrophen, adaptation occurred with a time constant of 1.3 ± 0.6 s (mean ± SD, n = 11; Györke and Fill, 1993). In the NP-EGTA experiments (Valdivia et al., 1995) the rate of adaptation (τ = 1.52 ± 0.2 s) was comparable, despite significant differences in Ca^{+2} spike kinetics (Ellis-Davies et al., 1996). Further, PKA-dependent phosphorylation increased the rate of RyR adaptation ~10-fold (Valdivia et al., 1995). The PKA-dependent phosphorylation would not be expected to alter the applied $[Ca^{+2}]_{FREE}$ waveform (i.e., the Ca^{2+} spike). Thus, the accumulated experimental data show that Ca^{2+} spike and RyR adaptation kinetics vary independently, indicating that the two phenomena are not causally related.

Attempts to relate Ca^{2+} spike and RyR adaptation kinetics is complicated by the dramatic temporal mismatch between the two phenomena, $\tau \approx 0.5$ and $\tau \approx 1300$ ms, respectively. Nevertheless, it was suggested that Ca^{+2} bound to the RyR during the Ca^{+2} spike activates the channel and that adaptation simply reflects the spontaneous closing of channels, as Ca^{+2} comes off the Ca^{+2} activation site(s) following the Ca^{+2} spike (Lamb and Stephenson, 1995). For this to be true, the RyR channel would have to deactivate very slowly in response to a fast $[Ca^{2+}]$ reduction. Intuitively, slow deactivation could be due to Ca^{2+} slowly coming off its activation site, to slow protein conformational changes following Ca^{2+} coming off the site, or to both phenomena.

To address these possibilities, here we define the response of single RyR channels to a fast $[Ca^{+2}]_{FREE}$ reduction, using photolysis of Diazo-2. We show that the channel deactivates with a time constant of 5.3 ms. Similarly, fast rates of RyR deactivation were reported by Sitsapesan et al. (1995) and Schiefer et al. (1995), who used mechanical solution change techniques. Thus, there is a good consensus that RyR deactivation is relatively fast. This means that it is very unlikely that RyR adaptation is due to slow deactivation following the Ca^{2+} spike.

Recently Schiefer et al. (1995) proposed that RyR channel gating can be explained by a simple state model in which the closed-to-open transition is Ca^{2+} dependent. The model of Schiefer et al. (1995) predicts that single RyR channels will respond rapidly and transiently (in <10 ms) to even the fastest Ca^{2+} signals. Thus the model of Schiefer et al. (1995) does not adequately describe the published experimental results (Györke and Fill, 1994; Valdivia et al., 1995). Future models could be improved by placement of the Ca^{2+}-dependent step between two closed states, effectively separating Ca^{2+} binding and channel opening. This relatively simple model constraint would still permit rapid channel activation but would not necessarily obligate the channel to respond to the fast Ca^{2+} spike.

Adaptation versus inactivation

The proposal that single RyR channels adapt, instead of inactivate, in response to a sustained $[Ca^{2+}]$ step (Györke and Fill, 1993) has motivated several groups of researchers to investigate Ca^{2+} regulation of single RyR channels. From these studies it is clear that the RyR channel has the

TABLE 1 Kinetics of single RyR channel Ca^{2+} regulation

	Experimental Laboratory				
Parameter	Györke and Fill (1993)	Valdivia et al. (1995)	Schiefer et al. (1995)	Laver and Curtis (1996)	Sitsapesan et al. (1995)
Species	Dog	Dog	Dog	Sheep	Sheep
Method	Photolysis, DM-Nitrophen	Photolysis, NP-EGTA	Mechanical, piezo-based	Mechanical, Puffing	Mechanical, Solenoid
Ca^{2+} stimulus	0.1 ⇒ 3.0 μM	0.1 ⇒ 10 μM	0.01 ⇄ 0.3–1000 μM	0.1 ⇄ 100 μM	0.1 ⇄ 100 μM
Ca^{2+} spike	Yes	Yes	No	No	No
Stimulus speed	<100 μs	<100 μs	≈1 ms	<20 ms	<10 ms
Activation	1.1 ms*	1.35 ms*	0.2–6.5 ms*	<20 ms*	<10 ms*
Deactivation	Not defined (5.3 ms,* see text and Fig. 2)	Not defined	6.0 ms*	<20 ms*	<10 ms*
Spontaneous decay of P_o	Yes	Yes	Yes	Yes	Yes, but infrequent
Decay rate	1.3 s*	1.4 s*	0.2–1.0 s*	2.0–15 s*	≈0.5 s*
Decay extent	Partial	Partial	Partial	Partial	Complete
Channel is refractory?	No ($[Ca^{2+}] \approx 1$ μm)	Not defined	No ($[Ca^{2+}] < 10$ μm)	Not defined	Not defined
Explanation of the decay	Adaptation	Adaptation modulated by Mg^{2+} and PKA	Inactivation with no Vm dependence	Unspecified	Vm and ATP dependent inactivation

*Values reported as time constants.

capacity (on and off kinetics) to track relatively fast $[Ca^{+2}]_{FREE}$ changes (Györke and Fill, 1993; Györke et al., 1994; Valdivia et al., 1995; Sitsapesan et al., 1995; Schiefer et al., 1995). Although there is good agreement concerning on/off kinetics, it is not yet clear whether channels adapt, inactivate, do neither, or do both. Results obtained in five different laboratories are summarized in Table 1.

Flash-photolysis studies of single canine RyR channels suggest that RyR channels adapt (Györke and Fill, 1993) and that adaptation is modulated by certain physiological ligands (Valdivia et al., 1995). Interestingly, the possibility that RyR channels adapt has been examined in vivo (Yasui et al., 1994; Rios, 1994; Györke and Györke, 1996). The negative control mechanism that regulates Ca^{2+} has properties that are consistent with adaptation. Thus, there are single-channel and whole-cell data supporting the adaptation proposal.

One mechanical solution change study showed that most single sheep RyR channels do not inactivate or desensitize in response to a $[Ca^{2+}]$ step (Sitsapesan et al., 1995). In the few channels that did inactivate (17%), the inactivation was voltage dependent and involved some sort of synergistic interaction of multiple ligands. Adaptive RyR channel behavior, however, was not observed when very large $[Ca^{+2}]_{FREE}$ stimuli (0.1 $\rightarrow$ 100 μM) were applied. However, adaptation in the photolysis studies was defined with much smaller $[Ca^{+2}]_{FREE}$ stimuli (0.1 $\rightarrow$ 1 μM). Sitsapesan et al. (1995) argue that a species difference in the Ca^{2+} sensitivity of sheep and canine channels may be responsible for the discrepancy. However, Laver et al. (1995) have recently shown that the steady-state Ca^{2+} sensitivity of the sheep channel is similar to that of the canine channel.

In contrast to Sitsapesan et al. (1995), Schiefer et al. (1995) showed that nearly all single canine RyR channels inactivate in response to a mechanically applied $[Ca^{2+}]$ step. The extent of the inactivation (Fig. 8 *B* of Schiefer et al., 1995) varied with $[Ca^{2+}]$ (less at pCa 6, more at pCa 3). Interestingly, the channels did not become "refractory," even when relatively large Ca^{2+} stimuli (10 μM) were applied at 20-ms intervals. This could indicate that the channels recover very quickly (<20 ms) from inactivation or that the channels never become refractory in the first place. At $[Ca^{2+}]$ values of <10 μM, desensitization without the channel becoming refractory would be consistent with single RyR adaptation (Györke and Fill; 1993) and reactivation of apparently desensitized Ca^{2+} release by tail currents in intact cardiac myocytes (Yasui et al., 1994).

The flash-photolysis and mechanical solution change methodologies have characteristic advantages and disadvantages. Photolysis ensures that Ca^{2+} is applied rapidly and uniformly without mechanical disruption. The disadvantages of photolysis include the fast Ca^{2+} spike and the production of photolytic by-products. The mechanical methods eliminate some of the disadvantages of photolysis but introduce new concerns about mechanical disruption, diffusion in unstirred layers (i.e., speed of stimulus), and what the channel actually "sees" in its microenvironment. The disparity between results from the flash-photolysis and the mechanical solution change studies are thus most likely due to methodological differences. As the methodological differences are resolved, the true nature of RyR channel Ca^{2+} regulation should be revealed.

Summary

The fast Ca^{+2} spike does not significantly activate single RyR channels reconstituted in planar lipid bilayers. Adaptation is not due to RyR deactivation following the fast Ca^{2+} spike. There is a good consensus concerning the on/off kinetics of the channel. A single RyR channel responds on a millisecond time scale to both elevations and reductions in $[Ca^{2+}]$. This is consistent with the observed properties of Ca^{2+} release in heart (Bers, 1991). Interestingly, there are studies that suggest that RyR channels adapt instead of inactivate, whereas other studies suggest that RyR channels inactivate instead of adapt. This disparity in results is likely due to methodological differences. Thus, resolution of the adaptation–inactivation question will clearly require more experimentation.

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