tion to transcriptional control circuits. The presence or absence of specific DNA bending proteins bound at their target sites would determine which neighboring binding sites for other proteins are favored.

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14. The strain used for β-galactosidase assays was *E. coli* JM002 {*araD139, ΔlacX74, galE, galK, phoA20, thy, rpsE, rpoB, argE*(*am*), [*mini Tn5-Km copG*]}, which is derived from *E. coli* CC118 [C. Manoil and J. Beckwith, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8129 (1985)]. It carries gene *copG* under the control of P_{tac} promoter, inserted into the chromosome with the use of a specialized transposon (K. Timmis and V. de Lorenzo, *Methods Enzymol.*, in press).
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Ryanodine Receptor Adaptation: Control Mechanism of Ca^{2+}-Induced Ca^{2+} Release in Heart

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Adaptation of single cardiac ryanodine receptor (RyR) channels was demonstrated by application of the caged calcium ion (Ca^{2+}) methodology. In contrast to conventional desensitization found in surface membrane ligand-gated channels, single cardiac RyR channels adapted to maintained Ca^{2+} stimuli, preserving their ability to respond to a second (larger) Ca^{2+} stimulus. RyR adaptation may represent a molecular control mechanism for smoothly graded Ca^{2+}-induced Ca^{2+} release in heart and may be a fundamental feature of channels, including the inositol trisphosphate receptor, that are involved in intracellular Ca^{2+} signaling in many cell types.

In cardiac muscle, Ca^{2+} release from the sarcoplasmic reticulum (SR) is mediated by a Ca^{2+}-activated channel called the ryanodine receptor (RyR) (*1–3*). The cardiac RyR is regulated by Ca^{2+} influx through voltage-gated Ca^{2+} channels in the surface membrane. This process, termed Ca^{2+}-induced Ca^{2+} release (CICR), is fundamental to cardiac excitation-contraction coupling, the mechanism that links surface membrane depolarization to Ca^{2+} activation of the contractile proteins (*4–8*).

In its simplest form, CICR should be an "all or nothing" phenomenon because of its intrinsic positive feedback. In vivo, however, CICR is smoothly graded (*5–7, 9*). To reconcile this paradox, Ca^{2+}-dependent inactivation was proposed as the essential negative control mechanism that counters the inherent positive feedback of CICR (*10*). Studies on intact and permeabilized cells present contradictory results concerning the existence of Ca^{2+}-dependent inactivation (*7, 10–12*). Also, single-channel studies done under steady-state conditions show no signs of inactivation at physiologically relevant Ca^{2+} concentrations (*13, 14*). In these single-channel experiments, however, it is possible that a regulatory subunit was lost during RyR isolation or that steady-state studies were inappropriate to describe a transient phenomenon.

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To directly define how CICR is controlled, we made calibrated changes in the Ca^{2+} concentration ($[Ca^{2+}]$) in the medium around single cardiac RyR channels from dog hearts by photolysis of caged Ca^{2+}. These experiments revealed a mechanism of Ca^{2+}-dependent adaptation of RyR channels that is distinct from conventional desensitization in that the channels adapt to a maintained Ca^{2+} stimulus and are thereby able to respond to subsequent Ca^{2+} stimuli. This adapting mechanism allows single RyRs to respond transiently to a Ca^{2+} stimulus in a dose-dependent manner and may represent a molecular control mechanism that underlies the smoothly graded nature of CICR in vivo.

Activation of a single cardiac RyR channel by flash photolysis of caged Ca^{2+} (*15*) is illustrated in Fig. 1A. To assure precise control of the $[Ca^{2+}]$ in the microenvironment of the channel, we used Cs^+ rather than Ca^{2+} as the conducting ion (*16–18*). The identity, sidedness, and number of the channels in the bilayer were determined under steady-state conditions (10 μM Ca^{2+}) before each experiment (*16–19*). We then added DM-nitrophen (caged Ca^{2+}) to the myoplasmic side of the RyR to buffer the $[Ca^{2+}]$ at 100 nM. At this $[Ca^{2+}]$, the stationary open probability was zero. Liberation of Ca^{2+} in the microenvironment of the channel by an ultraviolet (UV) flash (arrowhead) triggered a transient burst of channel activity (Fig. 1A). We reestablished resting conditions be-

tween UV flashes by stirring the medium for 30 s. We generated ensemble currents (Fig. 1B) by summing single-channel sweeps. We determined the time constant of activation (τ = 1.2 ms) by fitting a single exponential to the ensemble current. The activation rate of isolated RyR channels is consistent with the initial rate of CICR activation in vivo (*5–7*).

To further detail RyR activation, we examined the action of photolytically released Ca^{2+} on the activity of the RyR channel (Fig. 2A). Small increases in $[Ca^{2+}]$ activated the RyR channel in only a few instances. Thus, the result was a small ensemble current. Larger increases in $[Ca^{2+}]$ activated the channel more often, which resulted in larger ensemble currents. After a photolytic increase in $[Ca^{2+}]$, the probability of opening (P_o) peaked and then spontaneously decayed, whereas the $[Ca^{2+}]$ remained elevated (Figs. 1 and 2A) (*20*). This spontaneous decay could be related to Ca^{2+}-dependent inactivation (*10*). The rate of the decay (τ = 1.3 s) closely correlates with the rate of Ca^{2+}-dependent inactivation measured in permeablized cardiac myocytes (τ = 1.1 s; *10*). However, steady-state P_o measurements at constant bath $[Ca^{2+}]$ show no evidence of inactivation in the range of 0.1 to 10 μM $[Ca^{2+}]$ (Fig. 2B, open triangles) as described (*13, 14*).

To address this discrepancy, we compared the Ca^{2+} dependency of activation induced by caged Ca^{2+} photolysis (where P_o was measured at the peak of the ensemble currents) and the steady-state Ca^{2+} dependence of the channel (where P_o was measured at constant bath $[Ca^{2+}]$ over a long period of time). The channel's sensitivity to Ca^{2+} was approximately ten times greater when Ca^{2+} was applied by photolysis (Fig. 2B, open circles) than when Ca^{2+} was applied under steady-state conditions (Fig. 2B, open triangles). The steady-state measurements at constant $[Ca^{2+}]$ correlate directly with the Ca^{2+} dependence of P_o measured at the end of the photolytically induced ensemble currents (Fig. 2B, filled circles). These results suggest that the Ca^{2+} sensitivity of RyR activation decreases during prolonged exposure to Ca^{2+}. This phenomenon may account for the observed spontaneous decay of channel activity. A shift in Ca^{2+} sensitivity could explain why channels that apparently inactivate after fast increases in $[Ca^{2+}]$ remain active at much higher $[Ca^{2+}]$ under steady-state conditions.

To test if the spontaneous decay in channel activity resulted from a conventional mechanism (such as Ca^{2+}-dependent inactivation), we increased the $[Ca^{2+}]$ in two incremental steps (Fig. 3). A conventional

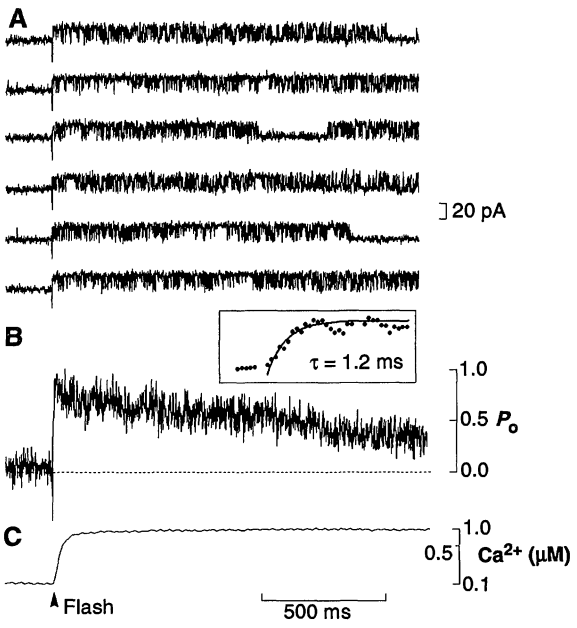

Fig. 1. Activation of single cardiac RyR channels by flash photolysis of caged Ca^{2+} (DM-nitrophen). Single-channel openings are shown as upward deflections. The current carrier was Cs^+. The UV laser flash was applied at the arrowhead. (**A**) Six independent examples of channel activation induced by flash photolysis of DM-nitrophen. (**B**) Ensemble current constructed from 87 data sweeps. (Inset) Time course of activation was best fit by a single exponential function (expanded scale). The time constant of activation was 1.1 ± 0.3 ms (n = 7; mean ± SD). (**C**) The amplitude and time course of the free Ca^{2+} change in the microenvironment of the channel was estimated in separate experiments in which the lipid bilayer was replaced with a Ca^{2+} ionophore resin (*20*). This transformed the bilayer aperture into a Ca^{2+} electrode while maintaining the geometric architecture of the experimental system.

mechanism predicts that if the channel becomes inactivated after the first increase in $[Ca^{2+}]$, it will not respond to the second increase in $[Ca^{2+}]$. However, application of two incremental increases in the $[Ca^{2+}]$ elicited two transient bursts of channel activity (Fig. 3). The cardiac RyR appears to adapt to the $[Ca^{2+}]$ to which it is exposed, preserving its capacity to respond to a new higher $[Ca^{2+}]$. Additionally, conventional desensitization at the single-channel level is characterized by bursts of openings separated by long silent intervals (*21*). In contrast, the opening frequency in the single-channel records appears to decrease gradually (Fig. 3A). Thus, the nature of our single-channel records is also inconsistent with conventional desensitization.

The spontaneous decay of channel activity, the shift in Ca^{2+} sensitivity, and the ability of apparently desensitized single channels to activate in response to a second Ca^{2+} stimulus support the hypothesis that RyR Ca^{2+} adaptation exists. We propose that the adaptation process includes the following events. (i) A change in $[Ca^{2+}]$ induces channel activity as a consequence of Ca^{2+} binding to an activation site on the RyR molecule. This step is relatively fast (τ ~ 1.2 ms; Fig. 1). (ii) The RyR molecule undergoes a transformation that induces a slow (τ ~ 1.3 s; Fig. 2) decrease in the Ca^{2+} affinity of the activation site. The result is less occupancy of the activation site and a decay in channel activity (relaxation phase). (iii) The relaxation phase leaves the activation site available to respond to a second Ca^{2+} stimulus (Fig. 3).

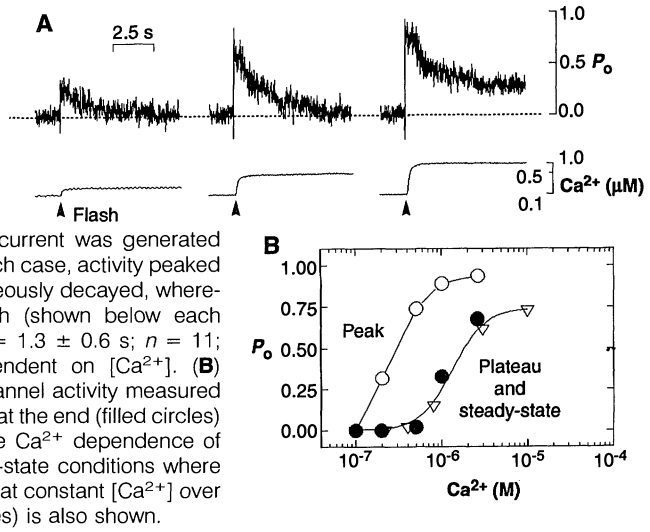

Fig. 2. Single-channel P_o measured during sustained Ca^{2+} stimuli. Free $[Ca^{2+}]$ before the flash was 100 nM. (**A**) Ensemble currents generated by the summation of 64 data sweeps at three concentrations of Ca^{2+} (0.2, 0.5, and 1 μM). Each ensemble current was generated from a different channel. In each case, activity peaked within 4 ms and then spontaneously decayed, whereas the $[Ca^{2+}]$ remained high (shown below each trace). The rate of decay (τ = 1.3 ± 0.6 s; n = 11; mean ± SD) was not dependent on $[Ca^{2+}]$. (**B**) Dependence on $[Ca^{2+}]$ of channel activity measured at the peak (open circles) and at the end (filled circles) of the ensemble currents. The Ca^{2+} dependence of channel activity under steady-state conditions where the average P_o was estimated at constant $[Ca^{2+}]$ over several minutes (open triangles) is also shown.

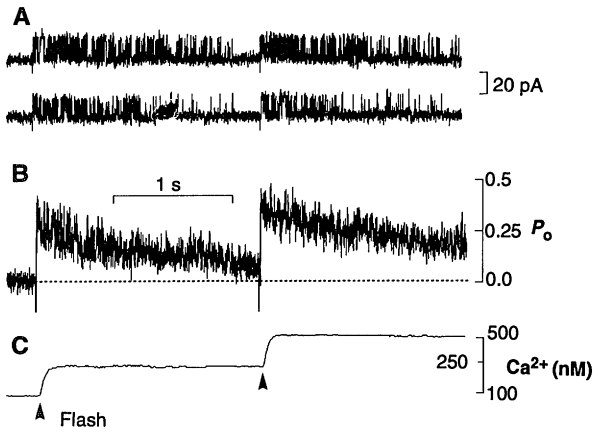

Fig. 3. Activation of the cardiac RyR channel by two incremental increases in [Ca^{2+}]. Two UV flashes were applied 2 s apart (at arrowheads). (**A**) Single-channel records. Open events are shown as upward deflections. (**B**) Ensemble currents generated from 92 data sweeps collected from a single channel (probability of multiple channels <0.0001) (*19*). (**C**) Time course of the change in [Ca^{2+}]. Data are representative of six experiments.

Cardiac RyR adaptation reconciles a body of apparently contradictory results. The fact that the cardiac RyR becomes insensitive only to the [Ca^{2+}] to which it is exposed explains why Ca^{2+}-dependent inactivation was evident in some studies (*10, 12*) and absent in others (*7, 11, 13, 14*). Cardiac RyR adaptation may represent the negative control mechanism that counters the inherent positive feedback of CICR. In our study, the rate of RyR adaptation appears to be too slow (10^3 times slower than RyR activation) to account by itself for the gradation of CICR in vivo. It is possible, however, that the rate of adaptation is faster under normal physiological conditions [in the presence of Mg^{2+}, adenosine triphosphate (ATP), and Ca^{2+} as the conducting ion]. Alternatively, even an adaptation with relatively slow kinetics could have a role in controlling CICR. Modeling studies have shown that if the Ca^{2+} sensitivity of the Ca^{2+} release mechanism is precisely adjusted, it is possible to obtain graded control of CICR (*22*). Such models, however, are intrinsically unstable and tend toward spontaneous oscillation (*22*). Adaptation could provide a mechanism to continuously fine-tune the Ca^{2+} sensitivity of the system to maintain a stable, graded CICR.

Single-channel adaptation may not be unique to the RyR. A similar mechanism may underlie the phenomenon of quantal or incremental Ca^{2+} release from inositol trisphosphate (IP_3)–sensitive Ca^{2+} stores by submaximal doses of IP_3 (*23–27*). The origin of quantal Ca^{2+} release from IP_3-sensitive stores has been a subject of debate. It has been attributed either to a heterogenous population of Ca^{2+} stores with different IP_3 sensitivities (*24, 27*) or to a gradual attenuation of IP_3 sensitivity in individual IP_3 receptors (IP_3Rs) (*28–30*). Our study demonstrates that RyR adaptation occurs in individual channels as a result of a shift in ligand sensitivity. Because the RyR and IP_3R are similar proteins (*31–33*), quantal behavior in IP_3R populations may also arise from individual IP_3Rs and result from modulation of the receptor sensitivity to IP_3. Thus, quantal Ca^{2+} release from IP_3R populations might be a consequence of adaptation of single IP_3R channels to incremental doses of IP_3.

As proposed for the IP_3R (*28*), a shift in ligand sensitivity might be regulated by a second Ca^{2+} binding site that interacts with the activation site in an allosteric fashion. Thus, adaptation may arise from allosteric interactions in the homotetrameric structure (*33*) of the RyR. Alternatively, it could be attributed to an interaction between the RyR and an unidentified, closely associated regulatory protein. A model based on the existence of a catalytic regulatory molecule that controls the transition between open and closed forms of the IP_3R channel has been proposed (*34*). Regardless of the specific mechanism, adaptation appears to be a fundamental feature of intracellular Ca^{2+} release channels, including both the RyR and IP_3R.

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15. Heavy SR microsomes were isolated by differential centrifugation from the ventricles of dog heart by standard procedures [C. A. Tate *et al.*, *J. Biol. Chem.* **260**, 9618 (1985)]. Microsomes were fused into planar lipid bilayers, and single channels were monitored as described (*10, 11*). Bilayers were composed of 70% phosphatidylethanolamine and 30% phosphatidylcholine in *n*-decane. Standard solutions contained 250 mM *cis*-$CsCH_3SO_3$, 50 mM *trans*-$CsCH_3SO_3$, 20 mM Hepes (pH 7.4), 3 mM DM-nitrophen (Calbiochem, San Diego, CA), 2 mM $CaCl_2$, and 2 mM glutathione. Bath [Ca^{2+}] was constantly monitored by a conventional Ca^{2+} electrode (World Precision Instruments, Sarasota, FL). The Cs^+-conducting RyR channels were sensitive to Ca^{2+}, Mg^{2+}, ATP, ryanodine, ruthenium red, and polyclonal antibodies to the RyR (*16, 17*). Channel sidedness was determined by the channel's response to ATP. The number of channels per experiment was determined during 5-min periods at 10 μM free Ca^{2+} (*19*). A fused silica single fiber optic with a 400-μm diameter was positioned 50 μm in front of the bilayer with a micromanipulator. The UV output of a Nd:yttrium-aluminum-garnet laser was focused on the other end of the optic fiber by a ×10 fused silica lens.
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20. We measured time course of local Ca^{2+} changes near the bilayer surface by transforming the bilayer chamber into a Ca^{2+} electrode by filling the bilayer aperture with a Ca^{2+} ionophore resin. Although the absolute rate of caged Ca^{2+} dissociation was not resolved ($\tau \sim 100$ ns) [J. H. Kaplan and G. C. R. Ellis-Davies, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6571 (1988)], the electrode followed slow (>100 ms) changes in [Ca^{2+}] accurately. The photolytic Ca^{2+} stimuli were essentially steps (Fig. 1C) because diffusion was physically restricted and is relatively slow (1 mm/10 min). This Ca^{2+} electrode was calibrated with Ca^{2+} standard solutions (Molecular Probes, Eugene, OR). Reliability of this method was confirmed with controls including sham flashes, addition of EGTA, rapid stirring, and fluorescence measurements (Rhod-2, Molecular Probes).
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