

Do Inactivation Mechanisms Rather than Adaptation Hold the Key to Understanding Ryanodine Receptor Channel Gating?

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The use of flash photolysis of caged Ca^{2+} to rapidly elevate $[\text{Ca}^{2+}]$ at the cytosolic face of a cardiac ryanodine receptor (RyR) channel reconstituted into a bilayer gives rise to a rapid increase in open probability (P_o ; Györke and Fill, 1993). This is followed by a decline in P_o , which occurs much more slowly ($\tau = 1.3$ s). After the decline in P_o , the channels can be reactivated by a second Ca^{2+} stimulus. These results led to the suggestion that RyR channels can adapt to a maintained Ca^{2+} stimulus. The report was of great interest for two main reasons.

First, the concept that Ca^{2+} -induced Ca^{2+} release (CICR) was a process that was smoothly graded and dependent on the magnitude of the trigger rather than an all-or-nothing process was difficult to explain. Not long before the publication of the Györke and Fill report (1993), Stern (1992) had brought this issue to the forefront of discussions on excitation–contraction (EC) coupling in cardiac muscle. He elegantly described how global models of EC coupling needed to be discarded because they were inherently unstable and that “local control” models, where discrete units of RyR channels were activated individually, could be operating. Stern (1992) argued that diffusion of Ca^{2+} away from the Ca^{2+} activation sites on RyR, coupled with the process of “stochastic attrition,” led to the inactivation of the Ca^{2+} release units. The contraction of the whole-cell, therefore, resulted from the summation of the individual packets of Ca^{2+} released from the separate release units. Stern’s local control model of cardiac EC coupling made sense of seemingly irreconcilable discrepancies and stimulated workers in the field to investigate, extend, or disprove his theory. It was at this time that Györke and Fill (1993) presented adaptation as a self-regulating property of RyR channels that could serve as a “molecular control mechanism for smoothly graded Ca^{2+} -induced Ca^{2+} -release in heart.” On the face of it, they had uncovered a mechanism that would close the RyR channels to allow reaccumulation of Ca^{2+} within sarcoplasmic reticulum (SR) stores, and that could explain why CICR was not an all-or-nothing positive feedback process. Györke and Fill’s paper (1993) was closely followed by the first report of Ca^{2+}

sparks by Cheng et al. (1993). The improved temporal and spatial resolution of in situ SR Ca^{2+} -release events afforded by the use of the confocal microscope accelerated the integration of theoretical models of EC coupling with experimental data. Here was evidence that local control of individual Ca^{2+} release units could provide smoothly graded CICR. This was an exciting time for cardiac physiologists and biophysicists, and the idea that adaptation of RyR might refine existing models of EC coupling was appealing.

The second reason for the interest in this work was the proposal that the phenomenon of adaptation was not merely the response of RyR to a rapid step change in $[\text{Ca}^{2+}]$, but that the Ca^{2+} spike that preceded the maintained increase in $[\text{Ca}^{2+}]$ itself affected channel gating (Lamb et al., 1994; Lamb and Laver, 1998). Thus, Lamb and co-workers (Lamb et al., 1994; Lamb and Laver, 1998) argued adaptation was the overall response of RyR channels to both the Ca^{2+} spike and the longer lasting increase in $[\text{Ca}^{2+}]$. The idea that the Ca^{2+} spike influenced the occurrence of adaptation was fueled by subsequent reports that adaptation did not occur when rapid changes in cytosolic $[\text{Ca}^{2+}]$ were produced using methods other than flash photolysis of a caged Ca^{2+} compound (Schiefer et al., 1995; Sitsapesan et al., 1995; Laver and Curtis, 1996). The issue was further confounded by the more recent report that Ca^{2+} spikes, in the absence of a maintained elevation in $[\text{Ca}^{2+}]$, could activate RyR channels (Zahradníková et al., 1999), although previously Györke and Fill (1994) had claimed vigorously that Ca^{2+} spikes could not activate RyR channels. In fact, this was used as evidence indicating that the fast Ca^{2+} spike did not influence the effect of the more maintained change in $[\text{Ca}^{2+}]$ (Györke and Fill, 1994).

There are certain issues that need to be resolved before we can understand how RyR channels respond to rapid changes in $[\text{Ca}^{2+}]$, and before we can hope to understand how SR Ca^{2+} release is triggered and terminated. First, does the Ca^{2+} spike that precedes the maintained increase in $[\text{Ca}^{2+}]$ in flash photolysis experiments affect the subsequent gating behavior of RyR? Second,

what is the response of the RyR channel to a maintained step increase in $[Ca^{2+}]$? Finally, does the gating behavior of RyR channels and the evidence from triggered Ca^{2+} release in intact cells support the proposal that RyR channels adapt to a maintained increase in $[Ca^{2+}]$?

Does the Ca^{2+} Spike that Precedes the Maintained Increase in $[Ca^{2+}]$ in Flash Photolysis Experiments Affect the Subsequent Gating Behavior of RyR?

The answer to this question is yes. Zahradníková et al. (1999) recently demonstrated, not surprisingly, that RyR channels are very sensitive to the amplitude of the $[Ca^{2+}]$ spike. Therefore, it is probable that if channels are first activated by a Ca^{2+} spike, the gating behavior of these channels in response to a subsequent maintained elevation in $[Ca^{2+}]$ will be different from the response of channels exposed solely to a step maintained elevation in $[Ca^{2+}]$. Therefore, we must examine how the channels respond to a step change in $[Ca^{2+}]$ in the absence of a preceding Ca^{2+} spike.

What Is the Response of the RyR Channel to a Maintained Step Increase in $[Ca^{2+}]$?

Three methods of imposing a step change in $[Ca^{2+}]$ have been used and all involve modifications of solution exchange protocols. All the methods produced a slower increase in $[Ca^{2+}]$ than the flash photolysis method, but all had the advantage of producing a step change in $[Ca^{2+}]$ to a maintained level. None of these methods gave rise to any suggestion of adaptation, but all three methods provided evidence that inactivation mechanisms could be induced if certain experimental conditions were met. The observed inactivation mechanisms, however, did not show any characteristics of adaptation. (We use the term "inactivation" to describe any reduction in P_o because experimental evidence indicates that this term is more appropriate than "desensitization." Although the cardiac RyR is a ligand-activated channel, gating is voltage-dependent [Sitsapesan and Williams, 1994]. Under certain conditions, ligand-activated RyR can be closed in an abrupt fashion and channel openings can only be restored by changing the transmembrane potential [Sitsapesan et al., 1995].) Nevertheless, some authors have ignored the differences between the flash photolysis experiments and the solution exchange methods in an attempt to unify any decline in P_o as a manifestation of adaptation (Vélez et al., 1997; Györke, 1999). Therefore, we will define the details of inactivation observed with the three solution exchange methods so that an objective comparison between adaptation and inactivation can be made.

Sitsapesan et al. (1995) made step changes in cytosolic $[Ca^{2+}]$ within 10 ms. The key observation of this report was that switching from 0.1 μM Ca^{2+} to a higher level in the absence of other channel activators, at either +40 or

-40 mV, normally did not elicit a decline in P_o after activation. This was observed for changes in $[Ca^{2+}]$ from 0.1 μM to either 1 (threshold for activation), 10, 50, or 500 μM . In 17% of the channels activated in this way, with Ca^{2+} as the sole ligand, an abrupt inactivation (no further openings) was observed at a holding potential of +40 mV. At -40 mV, no inactivation was observed. The inactivation observed at the positive holding potentials could only be reversed by a change in the polarity of the holding potential. In the absence of a change in polarity, switching out of the high Ca^{2+} solution back to 0.1 μM Ca^{2+} and then switching again to the higher $[Ca^{2+}]$ could not reverse the inactivation. This type of inactivation was much more frequent and pronounced when the channels were synergistically activated by Ca^{2+} plus a second ligand (either ATP or EMD 41000 [a caffeine analogue]) and occurred in 56% of the channels. In this series of experiments, the only decline in P_o after channel activation was an abrupt inactivation, which, in contrast to the flash photolysis experiments, while apparently use-dependent, was not reversed by changing the ligand concentration. Also important to bear in mind is the demonstration that the rapid increases in $[Ca^{2+}]$ (<10 ms) reported by Sitsapesan et al. (1995) did not elicit greater increases in P_o than slow changes in $[Ca^{2+}]$ (solution exchange time = ~ 1 s; Sitsapesan et al., 1995), and there was no evidence that channel gating behavior differed from that previously observed under steady state conditions (Sitsapesan and Williams, 1994). This contrasts with the results from the flash photolysis experiments.

Schiefer et al. (1995) accomplished solution exchanges within 1 ms. The experiments investigated the effects of switching from 0.1 μM Ca^{2+} to various higher levels of Ca^{2+} , but no other activating ligands were present. The experiments were all performed at a holding potential of +50 mV. As with the technique used by Sitsapesan et al. (1995), an inactivation mechanism was operating but, again, this mechanism had no common features with adaptation. In this respect, two critical observations were made. Adaptation in response to flash photolysis only occurs when very small changes in $[Ca^{2+}]$ are imposed (usually <1 μM). Figure 8 in the Schiefer et al. (1995) paper demonstrates that inactivation did not occur at such small increments in $[Ca^{2+}]$, and that inactivation actually increased with increasing $[Ca^{2+}]$. The highest degree of inactivation was observed at $[Ca^{2+}]$ levels at which adaptation is not seen. This was not the only piece of evidence indicating that adaptation and inactivation are irreconcilable hypotheses. A key feature of the adaptation theory is that even though the P_o of RyR channels declines after a small Ca^{2+} stimulus, the channels can continue to respond to subsequent increases in $[Ca^{2+}]$. Using the solution exchange method, Schiefer et al. (1995) demonstrated categorically that the channels become refractory to

subsequent increases in $[Ca^{2+}]$ after a time-dependent decrease in P_o , again reinforcing the evidence that inactivation, not adaptation is occurring.

Laver and Curtis (1996) produced step changes in cytosolic $[Ca^{2+}]$ within 20–110 ms. The experiments examined the effects of rapid activation of RyR channels with relatively high $[Ca^{2+}]$ (usually 100–200 μM) at a holding potential of +40 mV. In keeping with the well-known variability of the response of RyR channels to cytosolic Ca^{2+} , after the initial activation, a decline in P_o was observed in 70% of the RyR channels. However, the reduction in P_o , had the characteristics of inactivation rather than adaptation. Not only was the decline in P_o observed at the high $[Ca^{2+}]$ at which adaptation is reported not to occur, but as in the Schiefer et al. (1995) experiments, the higher the original P_o attained, the greater the decline in P_o (at least on the activating portion of Ca^{2+} - P_o dose-response relationship).

As mentioned earlier, a crucial difference between the flash photolysis method and the solution exchange methods is that flash photolysis gives rise to channel openings when $[Ca^{2+}]$ is increased from 0.1 μM to very low levels (<1 μM). Flash photolysis-induced increases in $[Ca^{2+}]$ above 10 μM do not give rise to adaptation (Györke and Fill, 1993). In contrast, the $[Ca^{2+}]$ threshold for channel activation in all the solution exchange methods was higher, 0.3–1 μM . If any subsequent decline in P_o was observed, such a decline was more likely, or of greater magnitude, as the concentration of activating Ca^{2+} was increased (Schiefer et al., 1995; Sitsapesan et al., 1995; Laver and Curtis, 1996). A likely explanation for the apparent greater sensitivity of the channels exposed to flash photolysis is that the initial Ca^{2+} spike, which usually reaches levels well in excess of 10 μM , provides the stimulus for the activation of the channels when the final steady state $[Ca^{2+}]$ reached is lower than the threshold required to activate channels in the solution exchange methods. Therefore, adaptation may be reduced as the maintained $[Ca^{2+}]$ is increased because the spike has a relatively smaller contribution to the overall P_o of the channel. Thus, the three different reports using the solution exchange methods provide no evidence that RyR channels adapt to a step change in $[Ca^{2+}]$, but, instead, indicate that inactivation does occur in a manner that is closely regulated by the experimental conditions.

Does the Gating Behavior of RyR Channels and the Evidence from Triggered Ca^{2+} Release in Cardiac Cells Support the Proposal that RyR Channels Adapt to a Maintained Increase in $[Ca^{2+}]$?

The flash photolysis experiments have catalyzed the development of numerous models for RyR channel gating. Adaptation is incorporated as a fundamental property of RyR function that can be used to explain how

Ca^{2+} -induced Ca^{2+} release functions as a smoothly graded process in cardiac muscle (Cheng et al., 1995; Sachs et al., 1995; Keizer and Levine, 1996; Villalba-Galea et al., 1998). However, by constraining the models to accommodate adaptation, crucial elements of RyR channel gating have been omitted. It has been suggested that modal gating of RyR channels allows the process of adaptation to proceed after a step change in $[Ca^{2+}]$ (Zahradníková and Zahradník, 1996). It was suggested that, at the onset of a rapid Ca^{2+} elevation, the channel could bind Ca^{2+} only in a high (H) P_o mode. Subsequently, the channel slowly cycles between H-mode, a low (L) P_o -mode, and an inactivated mode (I), thus, achieving the high initial P_o followed by a gradual decline in P_o with time. However, more recent work on cardiac RyR gating provides evidence that if the sequences of dwell times obtained from single-channel recordings are analyzed at multiple $[Ca^{2+}]$, the nature of the modal gating behavior predicts that adaptation would not occur in response to a step increase in cytosolic $[Ca^{2+}]$ (Saftenku et al., 2000). Open lifetime distributions at low $[Ca^{2+}]$ indicate that the channels are predominantly gating in the L-mode not the H-mode, as predicted from the Zahradníková and Zahradník (1996) model. Increases in cytosolic $[Ca^{2+}]$ actually result in an increase in P_o within L-activity and an increase in the probability of occurrence of H-activity, thus, providing no evidence that adaptation would occur in response to a rapid increase in cytosolic $[Ca^{2+}]$.

Just as the rapid solution exchange methods of increasing $[Ca^{2+}]$, together with a thorough examination of steady state RyR gating kinetics, argue against the likelihood that adaptation is really the response of RyR to a maintained step increase in $[Ca^{2+}]$ in bilayers, so whole-cell experiments also argue against the likelihood that adaptation is a mechanism that plays a role during EC coupling. Experiments with isolated cardiac myocytes demonstrate that after SR Ca^{2+} release has been triggered, the SR becomes refractory to the Ca^{2+} entering the cell through the L-type Ca^{2+} channels (Sham et al., 1998; Tanaka et al., 1998). Importantly, the RyR channels do not respond to L-type Ca^{2+} channel openings, even though the SR has not been fully depleted; therefore, the refractory period cannot simply be the result of depletion of the luminal Ca^{2+} stores (although a change in calsequestrin-mediated effects on RyR gating cannot be ruled out). Moreover, adaptation theory predicts that once the RyR channels have been activated, it should be possible to repeatedly reactivate the channels simply by imposing increments in $[Ca^{2+}]$ (Györke and Fill, 1993). However, Sham et al. (1998) demonstrate that this is unlikely. Even after applying a very large increment in $[Ca^{2+}]$ by applying depolarizing pulses to the cell followed by a hyperpolarizing pulse to activate a large tail current, the tail current activated only a small release of

Ca²⁺ that was likely due to the opening of RyR channels not previously activated by the depolarizing pulse. The results argue against an adaptive mechanism playing a role in the termination of SR Ca²⁺ release during EC coupling. It appears much more likely that inactivation processes are involved. But what are the inactivation mechanisms? Unfortunately, single-channel studies have not yet given us the answer to this question, although we have hints as to what they might be. The technical difficulties of the bilayer experiments coupled with the desire to unravel the basic mechanisms underlying RyR gating have led to a concentration on experiments designed to determine the effects of cytosolic Ca²⁺ in the absence of other ligands. The paucity of information on the mechanisms involved in the regulation of the gating of RyR by Ca²⁺ in the presence of other ligands, potential regulatory accessory proteins, or by the phosphorylation or redox state of the channel means that, at present, it is not possible to incorporate single-channel data into current models of EC coupling. All three solution exchange techniques for rapidly increasing [Ca²⁺] have provided evidence for inactivation processes that could be operational in the whole cell. Evidence indicates that inactivation mechanisms are often dependent on [Ca²⁺]; the higher the [Ca²⁺], the more probable or more rapid the inactivation (Schiefer et al., 1995; Laver and Curtis, 1996). It is also clear that many other conditions will affect the manner in which Ca²⁺ can inactivate the channel, e.g., the presence of a second ligand such as ATP (Sitsapesan et al., 1995). A detailed review of some of the other contributors to RyR channel inactivation and inhibition is provided by Lamb and Laver (1998).

In summary, since a maintained increase in [Ca²⁺] cannot be achieved using flash photolysis of caged Ca²⁺ without the production of an initial Ca²⁺ spike, and since Ca²⁺ spikes alone have been shown to activate RyR, it appears likely that the adaptation of RyR gating seen in such experiments is the result of the combined effects of the Ca²⁺ spike plus the more maintained increase in [Ca²⁺]. This is confirmed by the fact that inactivation, not adaptation, is observed by three independent groups using different solution-exchange methods of rapidly increasing [Ca²⁺]. There is also growing evidence that termination of SR Ca²⁺ release in situ must result from strong inactivation of RyR channels rather than adaptation. Therefore, it would be logical to concentrate on investigating what the mechanism or mechanisms of inactivation could be, and how they are switched on and off. Inactivation in situ may result from the effect of a single regulatory ligand or, alternatively, (and more likely) multiple factors may be involved. For example, ATP may play a role in triggering inactivation as physiological levels of this ligand have been shown to inactivate the channel in a Ca²⁺-dependent manner (Kermode et al., 1998). Other candidates with a possi-

ble role in channel inactivation include calmodulin, Mg²⁺, calsequestrin, and the luminal [Ca²⁺]. Further investigation and integration of RyR channel gating and of the mechanisms terminating SR Ca²⁺ release in cardiac myocytes, therefore, is required before a more refined model of cardiac EC coupling can be achieved.

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