Perspective

Questions about Adaptation in Ryanodine Receptors

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The proposal of adaptation in individual ryanodine receptor (RyR)/Ca^{2+} release channels (Györke and Fill, 1993) has been an exciting concept that has stimulated widespread consideration of the properties and mechanisms involved in the activation and termination of Ca^{2+} release in muscle and other cells. Here, we consider what adaptation of individual RyRs is defined to mean and examine the evidence for its existence in the light of the recent reappraisal of the stimulus used in the original experiments. We further discuss the findings of other single-channel studies, as well as in vivo studies on Ca^{2+} release designed to test a possible role of adaptation, and conclude that there are inconsistencies in the adaptation model of RyR regulation. In an attempt to resolve the issue, and to obtain insights into the fascinating dynamic responses of a RyR to large, transient Ca^{2+} stimuli, which may be fundamental for RyR function in vivo, we present an alternative model to explain the data of Györke and colleagues.

Adaptation in Individual RyRs and Ca^{2+} Spikes

Adaptation in individual RyRs should not be confused with the response displayed by a population of RyRs in situ, which can show an overall adaptation-like behavior by means of any of a number of mechanisms unrelated to adaptation of individual RyRs, e.g., recruitment and inactivation of subpopulations of RyRs; or regulation of RyRs by Ca^{2+} in the lumen of the sarcoplasmic reticulum (Lamb and Laver, 1998). Györke and Fill (1993) used the term adaptation to describe the behavior of individual RyRs in lipid bilayers when the $[Ca^{2+}]$ was rapidly raised by flash photolysis of DM-nitrophen, a type of caged Ca^{2+}. After the flash, the open probability (P_o) of the RyR rapidly increased to a high level before declining to a low level again over a few seconds and P_o could be increased immediately again by raising the $[Ca^{2+}]$ further with a second flash. Assuming that the $[Ca^{2+}]$ rose in a step-like manner to a new constant level, Györke and Fill (1993) proposed that the RyR was first very potently activated by the small increase in $[Ca^{2+}]$ (from 100 to 200 nM), and then "adapted" to this new level. Thus, the term "adaptation" refers to a specific phenomenon in individual RyRs, in which the RyR is activated to a relatively high open probability

(>0.3) by rapid application of a low $[Ca^{2+}]$ (200 nM), and then P_o decreases over ~ 1 s without the channel becoming refractory to another stimulus (i.e., the channel is not "inactivated").

After its initial description, we questioned the existence of adaptation. Our concern was that flash photolysis of nitrophen causes the $[Ca^{2+}]$ to transiently rise to a very high level (60 µM) before declining (within ~ 1 ms) to the final step level; thus, it was possible that this Ca^{2+} spike substantially influenced the subsequent gating behavior of the RyR (Lamb et al., 1994; Lamb and Stephenson, 1995; Lamb, 1997). Moreover, the rapid activation of the RyR (time constant of activation ~ 1 ms for step to 1 µM Ca^{2+}; Györke and Fill, 1993) would have implied a physically unrealistic apparent rate constant of association for Ca^{2+} binding to the RyR ($\geq 10^9$ $M^{-1}s^{-1}$), suggesting that the Ca^{2+} spike was driving activation. Initially, the existence of Ca^{2+} spikes was not accepted (Györke and Fill, 1994) and it was reported that Ca^{2+} spikes do not activate the RyR (Velez et al., 1997); but, more recent experiments show that the Ca^{2+} spike that occurs in the flash photolysis experiments drives the initial RyR activation (Zahradníková et al., 1999). Flash stimuli of relatively low energy elicit Ca^{2+} spikes that induce only brief (~ 5 ms) activation of the RyR, whereas stronger intensity flashes induce activity lasting ~ 1 s, the adaptation response (Zahradníková et al., 1999). However, it is not clear whether or not the Ca^{2+} spike causes the whole adaptation phenomenon. In other words, can a small rapid rise in $[Ca^{2+}]$ by itself potently activate the RyR, and does the RyR genuinely adapt to this stimulus such that it can be reactivated subsequently by a larger rise in $[Ca^{2+}]$? Alternatively, does the Ca^{2+} spike cause the increase in P_o, which in some circumstances takes ~ 1 s or more to return to a steady-state level determined by the final $[Ca^{2+}]$?

Does a Small, Rapid Rise in $[Ca^{2+}]$ by Itself Cause Potent Activation of a RyR?

As the Ca^{2+} spike elicited in the flash photolysis experiments drives the initial activation of the RyR (Zahradníková et al., 1999), the original data of Györke and Fill (1993) offer no clear evidence that a rapid increase in $[Ca^{2+}]$ to a level that is too low to cause appreciable acti-

vation of the RyR at steady state (e.g., 200 nM) can cause potent activation (and subsequent adaptation). Thus, it becomes necessary to revisit the problem and there are a number of observations from various studies that suggest that a small rapid increase in $[Ca^{2+}]$ by itself does not cause potent, prolonged (i.e., $\sim$1 s) activation of RyRs.

First, the presence of the prolonged activity is not well correlated with the size of the $[Ca^{2+}]$ step (i.e., the steady $[Ca^{2+}]$ after the Ca^{2+} spike). The recent flash photolysis study of Zahradníková et al. (1999) found that a rapid rise in $[Ca^{2+}]$ from 161 nM to a final level of 268 nM caused only brief activation ($\sim$5 ms) of the RyR and did not induce prolonged ($\sim$1 s) activity on any occasion (see the 27-μM spike case in Figure 4 C of that paper; the initial and final $[Ca^{2+}]$ values were provided by A. Zahradníková). This finding is difficult to reconcile with the notion that rapid step rises in $[Ca^{2+}]$ to $\sim$200–250 nM cause a large and prolonged (i.e., $\sim$1 s) increase in P_o. Furthermore, the size of the $[Ca^{2+}]$ step in this and other photolysis experiments is possibly an underestimate, owing to difficulties in measuring the changes in $[Ca^{2+}]$ in such experiments (see *Problems of [Ca²⁺] Measurements*).

Second, when a RyR is activated repetitively by a small $[Ca^{2+}]$ step in the flash photolysis experiments (a Ca^{2+} spike–step stimulus), it does not respond similarly to each stimulus, but rather responds in a seemingly all-or-none manner, showing prolonged high P_o activity on some occasions and no (or little) activity on other occasions (Györke and Fill, 1994). For example, steps to 200 nM Ca^{2+} elicit vigorous activity ($P_o > 0.6$) on some repetitions and no activity on others. Such behavior is not easily reconciled with the step rise in $[Ca^{2+}]$ being the stimulus, because even though it might not activate the channel immediately (i.e., within $\sim$10 ms), it would be expected to cause some channel activity over the next 100–500 ms, before the channel could have adapted to the increase in $[Ca^{2+}]$ (time constant $\sim$ 1 s). In contrast, the brief initial Ca^{2+} spike would be expected to elicit such an all-or-none behavior (see later).

Third, when true steps in $[Ca^{2+}]$ were applied to cardiac RyRs by a solution change method that did not induce any initial Ca^{2+} spike (Schiefer et al., 1995), channel activation was well described by Ca^{2+} binding with an on-rate of 2.3×10^8 $M^{-1}s^{-1}$ to a single type of site with an apparent K_d of $\sim$0.56 μM (Schiefer et al., 1995). The lowest $[Ca^{2+}]$ examined in that study was $\sim$320 nM; by extrapolation the P_o at 200 nM Ca^{2+} should be <0.1. There was no evidence for activation sites with an affinity sufficiently high to explain potent activation by genuine step (i.e., spike-free) increases in $[Ca^{2+}]$ to 200 nM.

Fourth, if the prolonged activity in the flash experiments were due solely to the final step $[Ca^{2+}]$ (i.e., the Ca^{2+} spike did not contribute to the prolonged activity), one would expect flash stimuli to produce two distinct,

independent kinetic components in the response of the RyR. The first would be due to activation–deactivation caused by the large, but brief, initial Ca^{2+} spike; the second would be due to the subsequent prolonged activation by the step stimulus. Specifically, if a RyR always deactivated quickly after a Ca^{2+} spike, as was found with small brief Ca^{2+} spike stimuli (P_o decay time constant $\sim$3.2 ms; Zahradníková et al., 1999), the P_o should peak at some high level before declining in <10 ms to a lower level determined by the putatively sensitive response to the final step $[Ca^{2+}]$. In fact, submaximal flash stimuli cause the mean P_o to peak within $\sim$1 or 2 ms (to a range 0.3–0.7), and then to decay slowly to zero (or a low level) without any rapid ($\sim$5–10 ms) phase of decline (see Figure 3 in Györke and Fill, 1993; Figure 6 in Györke et al., 1994; Figure 3 in Zahradníková et al., 1999). Thus, there is no obvious transition between the initial Ca^{2+} spike-induced activation and the putative step-induced activation, which argues against the notion that the spike and the final step increase in $[Ca^{2+}]$ activate the channel through independent processes. These findings are more easily reconciled with the idea that the Ca^{2+} spike (or spike and step together) cause the activity, as we discuss in more detail later.

Activation by Ca²⁺ Spike/Step Stimuli in Flash Photolysis Experiments

To proceed, we need to consider how Ca^{2+} spikes arise and how their size and duration depend on experimental conditions. As nitrophen has a high affinity for Ca^{2+} ($K_d \sim$5 nM), when the free $[Ca^{2+}]$ is $\sim$100 nM, most of the nitrophen has Ca^{2+} bound and the proportion of free nitrophen is quite low (e.g., $\sim$140 μM free out of a total of 3 mM nitrophen). Flash photolysis of nitrophen converts it to a form with very low affinity for $[Ca^{2+}]$ ($K_d \sim$3 mM). To increase steady $[Ca^{2+}]$ two fold (to 200 nM), a flash must photolyze sufficient nitrophen to result in an approximately two-fold decrease in the concentration of free nitrophen (to $\sim$70 μM). As most of the photolysed nitrophen has Ca^{2+} bound initially, the flash causes the liberation of a large amount of Ca^{2+} (i.e., $\sim$70 μM), virtually all of which is rapidly rebound by free nitrophen, resulting in the twofold decrease in its concentration (Lamb and Stephenson, 1995). (Similarly, if the $[Ca^{2+}]$ is raised ten-fold from 100 nM to 1 μM, there is only $\sim$14 μM free nitrophen remaining to buffer the Ca^{2+}). As a first approximation, the peak size of the Ca^{2+} spike will be proportional to the amount of nitrophen photolysed, and its duration will be inversely proportional to the amount of free nitrophen (and, hence, approximately proportional to the free $[Ca^{2+}]$) present before the flash. The important point is that a flash of a particular intensity will produce a Ca^{2+} spike of similar peak size but
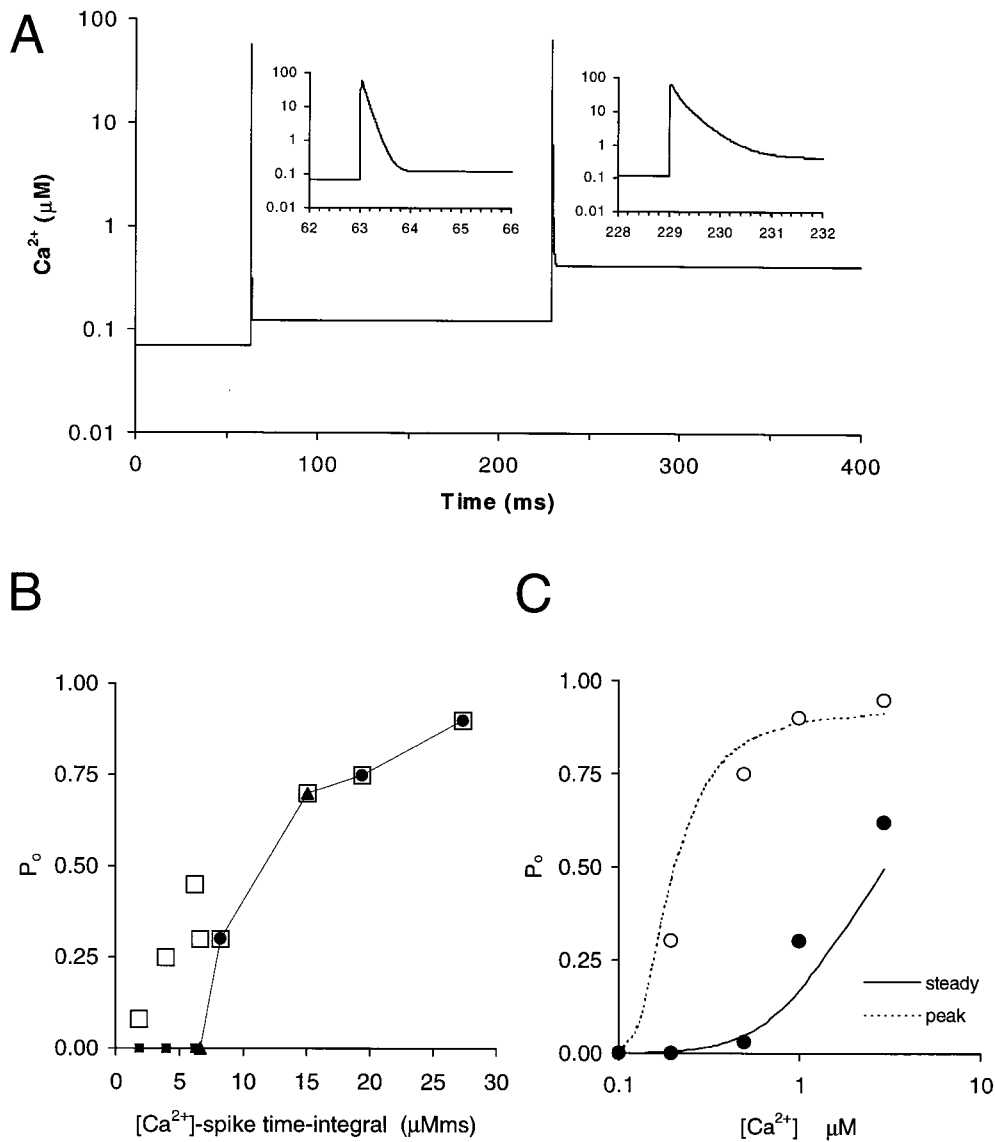
FIGURE 1. (A) Time course of Ca^{2+} spikes elicited in the double flash experiment of Zahradníková et al. (1999) (their Figure 3 A; and see published correction). The insets show that the second Ca^{2+} spike is much more prolonged than the first and, consequently, that its time integral is considerably larger. (B) Plot of the initial P_o (open symbols) and P_o after ~20 ms (closed symbols) versus the time integral of the Ca^{2+} spike for the flash photolysis data of Zahradníková et al. (1999) and Györke and Fill (1993). For each flash stimulus there is an open symbol and a corresponding closed symbol plotted against the same time integral value. Where a closed symbol is on the abscissa, the P_o dropped from the value indicated by the corresponding open symbol to zero within ~20 ms (i.e., the RyR gave only a brief response). Where open and closed symbols are superimposed, the P_o remained elevated over the first ~20 ms (i.e., the RyR gave a prolonged response). Closed squares relate to single flashes in Figure 4 (A–C) in Zahradníková et al. (1999) (starting $[Ca^{2+}]$ 161 nM to final levels of 186, 220, and 268 nM, provided by A. Zahradníková) and closed triangles relate to the pair of flashes in Figure 3 of the same study. Closed circles relate to Figure 2 of Györke and Fill (1993) (P_o of ~0.3, 0.75, and 0.9 for steps from 100 to 200, 500, and 1,000 nM Ca^{2+}). (C) Initial and steady-state P_o (dashed and solid lines, respectively) predicted to be elicited by the Ca^{2+} spike and step induced by flash photolysis when raising the $[Ca^{2+}]$ from 100 nM to the level indicated on the abscissa, assuming that the probability of RyR activation depends on Ca^{2+} binding to four sites, each with the k_{on} and k_{off} found by Schiefer et al. (1995). Open and closed symbols plot the observed initial and steady-state P_o obtained by Györke and Fill (1993; see their Figure 2 B).

longer duration if applied at higher $[Ca^{2+}]$ (and the same total nitrophen concentration; Fig. 1 A).

This becomes important because when considering the ability of a Ca^{2+} spike to activate a RyR, it is necessary to take into account its duration as well as its peak size. Ashley and Moisescu (1972) analyzed the general problem and showed how for any site S described by:

$$S + Ca^{2+} \underset{k_{off}}{\overset{k_{on}}{\leftrightarrow}} Ca^{2+}S$$
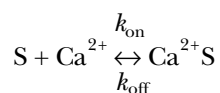
the probability ($P(t)$) that the site will be occupied by Ca^{2+} (i.e., be in state $Ca^{2+}S$) at time t depends on the time integral ($Int_{Ca}(t)$) of the Ca^{2+} stimulus ($Ca^{2+}(t)$) and can be expressed as:

$$P(t) = 1 - \exp[-(k_{on} \cdot Int_{Ca}(t) + k_{off} \cdot t)] - \varepsilon, \quad (1)$$

where ε is a correction factor that also depends on $Int_{Ca}(t)$ and which is small (<0.1) for the Ca^{2+} stimuli during the Ca^{2+} spike. Eq. 1 shows that it is the time integral of the Ca^{2+} spike, rather than its peak size alone, that determines Ca^{2+} binding to the RyR during activation.

We can now turn to the flash photolysis experiments in Zahradníková et al. (1999). These authors analyzed the brief activation of the RyR by low intensity flashes in

relation to the peak size of the Ca^{2+} spike (Figure 6 F in Zahradníková et al. 1999). They also tested whether the Ca^{2+} spike was inducing the prolonged (adaptation) response by performing flash photolysis with two successive, equal intensity flashes, 170-ms apart (Figure 3 in Zahradníková et al., 1999; and see published correction). In the latter experiment, the two flashes elicited Ca^{2+} spikes of similar peak size (Fig. 1 A), with the first spike/step causing only brief activation of the RyR on some occasions (mean P_o ~0.3 before decaying to zero within ~5–10 ms), and the second spike/step eliciting a prolonged (~1 s) high P_o (~0.7) response on most occasions. The data for the first flash (spike peak ~52 μM and P_o ~0.3) does not fit with the other data in Figure 6 F of Zahradníková et al. (1999; expected P_o ~0.8), because the first flash was done at a relatively low initial $[Ca^{2+}]$ (~70 nM), meaning that there was a relatively large amount of free nitrophen present initially, which caused the Ca^{2+} spike to be relatively brief (Fig. 1 A, first inset). When plotted against the time integral of the Ca^{2+} spike, the P_o value for the first flash in that double flash experiment agrees quite well with the other P_o values for brief activation obtained with different initial conditions (four leftmost open symbols in Fig. 1 B). In other words, the time integral (and not the peak) of the Ca^{2+} spike is a good predictor of the P_o of the brief activity, irrespective of the specific initial conditions.

Zahradníková et al. (1999) concluded that prolonged activity (adaptation behavior) of an RyR is determined exclusively by the final step $[Ca^{2+}]$ because both flashes in the double flash experiment elicited a Ca^{2+} spike of similar peak size, but only the second flash produced the prolonged activity. However, as discussed above, the Ca^{2+} spike produced by the second flash will be considerably longer than that produced by the first flash and the time integral of the second Ca^{2+} spike will be ~2.2 times larger than that of the first spike (Fig. 1 A). As the two Ca^{2+} spike stimuli were not identical, one cannot be sure that it was the final steady $[Ca^{2+}]$ level alone that was driving the RyRs into the prolonged high activity.

When one plots the initial P_o from the single and double flash experiments of Zahradníková et al. (1999) together with the initial P_o data from Györke and Fill (1993; Fig. 1 B, open symbols), there is a simple monotonic relationship between the initial open P_o and the time integral of the Ca^{2+} spike. Thus, the initial P_o level appears to be determined by the time integral of the Ca^{2+} spike, irrespective of whether the P_o declined rapidly (in ~5 ms) or was the start of a more prolonged response that declined over ~1 s or so (i.e., the adaptation response). The closed symbols in Fig. 1 B show the value of P_o after ~20 ms for the same experiments, with a symbol on the abscissa indicating that the P_o dropped quickly to zero (i.e.,. there was only a brief response),

and superposition of open and closed symbols indicating that the P_o remained elevated for some time. (Data from flash photolysis using a different Ca^{2+} cage [NP-EGTA; Valdivia et al., 1995] also fits with this apparent relationship, though the data are limited to only relatively large or very small spikes.) Based on this analysis, we conclude that the time integral of Ca^{2+} spike is a better predictor of the occurrence of prolonged high P_o activity than is the $[Ca^{2+}]$ reached by the step, and propose that once the time integral of the Ca^{2+} spike exceeds a critical level, the probability of initiating prolonged activation (and hence mean P_o) increases progressively (see closed symbols). This would mean that the Ca^{2+} spike is a primary factor not only for inducing the brief activation of the RyR, but also for inducing the prolonged P_o behavior (adaptation response).

If the Ca^{2+} spike affects the overall response of the RyR, one would expect the activity to show all-or-none behavior with near threshold stimuli (see *Does a Small, Rapid Rise...*), because the brief Ca^{2+} spike would be just sufficient to cause binding of Ca^{2+} to the one or more critical sites on some occasions but not on others. When the flash intensity is increased, the Ca^{2+} spike would get larger and longer in duration, and would drive Ca^{2+} onto the critical site(s) on most if not all occasions, which is consistent with the finding that stronger flashes induced the prolonged high P_o activity on almost every repetition (Györke and Fill, 1993). Ca^{2+} spikes of the size and time course occurring in the flash photolysis experiments for step rises from 100 to 200 nM Ca^{2+} (spike peak ~50 μM, decay constant ~ 0.2 ms) would produce Ca^{2+} binding to sites like those described by Schiefer et al. (1995) on ~85% of flashes. Thus, the Ca^{2+} spikes are of the very size and duration necessary to explain the all-or-none aspect of the observed channel behavior. One can plot the predicted initial and steady-state P_o values that would be elicited by the Ca^{2+} spike generated in the flash photolysis experiments of Györke and Fill, (1993) when raising the $[Ca^{2+}]$ from 100 nM to different final step levels (Figure 1 C) by assuming that the probability of RyR activation depends on Ca^{2+} binding to four sites (as proposed by Zahradníková et al., 1999), each with the k_{on} and k_{off} found in the solution change experiments by Schiefer et al. (1995). Such a plot seems to predict both the initial and steady P_o values found by Györke and Fill (1993) (open and closed symbols respectively, taken from their Figure 2), suggesting that the initial level of activation that they observe at each flash is well explained by the integral of the Ca^{2+} spike it elicits.

Ca^{2+} Spikes and the Progression from Brief to Prolonged Activation

Examination of Fig. 1 B suggests the response to Ca^{2+} spike stimuli might be as follows. If the Ca^{2+} spike is

comparatively small or brief, and, hence, has a comparatively small time integral, it induces only transient activation of the RyR on 10–30% of occasions (i.e., mean P_o 0.1–0.3), with the proportion of successful activations (and hence the mean P_o) increasing in a graded manner with the spike time integral (Fig. 1 B). If the Ca^{2+} spike is high or long enough, it could reach a point where the RyR moves into, or becomes locked into, a prolonged activity state for about a second. The exact basis for this effect is unclear, because there are presently no other data characterizing the dynamic response of the RyR to such transient stimuli. One possibility is that the Ca^{2+} spike helps drive the RyR into a special high P_o mode, from which it escapes slowly (i.e., $\sim$1 s). Such a mode (called H-mode) has been identified in cardiac RyRs in the presence of 5–50 μM steady-state $[Ca^{2+}]$ (Zahradníková and Zahradník, 1995), and it is noteworthy that, after entering this mode, the RyR typically stayed in it for $\sim$1 s. Thus, it is quite possible that the prolonged activity (adaptation response) observed in the flash photolysis experiments reflects the movement of the RyR into H-mode activity (Györke, 1999). As the RyR supposedly stays in this H-mode for $\sim$1 s, even though the $[Ca^{2+}]$ had declined to a low level (e.g., 200 nM) following the initial Ca^{2+} spike, the time to drop out of H-mode should be independent of the prevailing $[Ca^{2+}]$ because it was the same at both 200 nM Ca^{2+} (photolysis experiments) and $\geq$5 μM Ca^{2+} (Zahradníková and Zahradník, 1995).

Other experiments show that when the $[Ca^{2+}]$ is rapidly decreased from one steady level to a lower level, the RyR rapidly deactivates (Sitsapesan et al., 1995; Schiefer et al., 1995; Velez et al., 1997). These observations have been interpreted as arguing against the possibility that the Ca^{2+} spike drives the RyR into a state of prolonged activation. However, there is no consensus about what level or type of activity constitutes the H-mode, which makes it unclear whether it is possible to rapidly deactivate a RyR when it is in the H-mode simply by decreasing the $[Ca^{2+}]$. Certainly, no solution change experiments have been performed that specifically mimic the Ca^{2+} stimulus occurring in the flash photolysis experiments to test whether a RyR can be rapidly deactivated by lowering the $[Ca^{2+}]$ immediately (e.g., <1 ms) after the channel has been activated to a relatively high P_o by a high $[Ca^{2+}]$ stimulus. Nevertheless, it is easy to understand how a Ca^{2+} spike could have very peculiar effects because the Ca^{2+} spike is just large and wide enough to cause substantial occupancy of some activation sites on the RyR without affecting Ca^{2+} sites that do not have very high on-rates. Consequently, if the behavior of the RyR is influenced by its interaction with an associated Ca^{2+}-binding molecule (perhaps calmodulin) or by Ca^{2+} binding to other sites on the RyR itself, the Ca^{2+} spike stimulus could induce a unique state where Ca^{2+}

is bound to one type of site/molecule and not the other, a state which would not occur with more prolonged or steady-state Ca^{2+} stimuli. This could also explain why the ability of RyRs to show adaptation is apparently lost when they are purified (Velez et al., 1995).

Adaptation Versus Inactivation

In addition to heightened sensitivity to small $[Ca^{2+}]$ steps, the other major feature of adaptation reported in the flash photolysis experiments of Györke and Fill (1993) is that the activity (P_o) of a RyR stimulated by a spike/step to a final $[Ca^{2+}]$ in the range 200 nM to 1 μM progressively declined over $\sim$1 or 2 s, but could be increased immediately again by a second spike/step to a higher final $[Ca^{2+}]$ (i.e., the RyR was not refractory). Such adaptation is not observed in experiments where the $[Ca^{2+}]$ is rapidly raised (without any Ca^{2+} spike) by solution exchange (flow) methods (Schiefer et al., 1995; Sitsapesan et al., 1995; Laver and Lamb, 1998): P_o either remained constant or, in cases where it did decline, it was attributed to inactivation of the RyR,[1] a process which by definition is different from adaptation. If an RyR is inactivated, it cannot be reactivated immediately by applying a larger stimulus. This is the fundamental difference between adaptation and inactivation that led Györke and Fill (1993) to use the term adaptation to describe their results. Schiefer et al. (1995), who like Györke and colleagues used canine cardiac RyRs, found no significant decline in P_o for $[Ca^{2+}]$ steps to final levels of 320 nM and 1 μM. When using considerably higher $[Ca^{2+}]$ steps (to 3 μM–1 mM), Schiefer et al. (1995) found two forms of inactivation, in which the mean P_o declined with a time constant of 0.2 to 1 s. It is unclear whether the inactivation occurring with $[Ca^{2+}]$ steps to 3–30 μM could be adaptation because there was no examination of whether an inactivated RyR could be reactivated by still higher $[Ca^{2+}]$. It is also unclear whether the decline in P_o at these high $[Ca^{2+}]$ was due to a progressive decrease in P_o during the course of each stimulus or to a complete cessation of activity on some repetitions and not others, as occurred at higher $[Ca^{2+}]$ in the same

[1]"Inactivation" and "desensitization" are names traditionally used to describe refractory behavior in voltage- and ligand-gated channels, respectively. Laver and Lamb (1998) found that two RyR phenomena arise out of one inactivation mechanism: (1) the channel becomes refractory to the continued presence of a stimulatory ligand (i.e., desensitization); and (2) the channel activity declines after a voltage-step (inactivation). Thus, both terms ("desensitization" and "inactivation") could be used to describe the one mechanism depending on how it is observed (ligand-step or voltage-step). Although RyRs are gated by both voltage and by ligands, "inactivation" has generally been adopted as the term to describe refractory closing of RyRs. Inactivation appears as long closed periods that separate spontaneous bursts of activity. During a closed period, the RyR cannot be activated by raising the ligand concentration but can be reactivated immediately by changing the voltage (see Figure 10 in Laver and Lamb, 1998).

study (Schiefer et al., 1995). In summary, Schiefer et al. (1995) found no evidence of adaptation to small [Ca^{2+}] ($\leq$1 μM) steps, the crucial range for the phenomenon in the photolysis experiments, and describe the decline in P$_o$ that they observed with large [Ca^{2+}] steps ($\geq$3 μM) as inactivation though it was only convincingly shown to be inactivation for steps to >30 μM Ca^{2+}.

Sitsapesan et al. (1995), using sheep cardiac RyRs, and Laver and Lamb (1998), using rabbit cardiac and skeletal RyRs, found that the response to sustained [Ca^{2+}] steps declined with a time constant of 1–10 s in approximately half of the RyRs examined. In cases where a decline did occur, it depended on the membrane potential and on the P$_o$ itself, occurring only if the RyR activity was high. This decline in activity is fundamentally different from adaptation because of the following: (1) the RyRs could not be reactivated by either repeated [Ca^{2+}] steps from low to high [Ca^{2+}] (Sitsapesan et al., 1995) or sequential step increments in [Ca^{2+}] (Laver and Lamb, 1998); (2) the decline in activity did not depend specifically on the Ca^{2+} stimulus, but also occurred in channels activated by ryanodine or ATP plus caffeine; and (3) its kinetics features were quite different from adaptation, involving the sudden onset of long lasting closures, whereas with adaptation P$_o$ often appears to decline incrementally during each stimulus repetition. Similar voltage-dependent inactivation was observed by Zahradníková and Meszaros (1998) in skeletal RyRs. This voltage- and P$_o$-dependent inactivation, which does not require Ca^{2+} binding per se, should not be confused with the "Ca^{2+} inactivation" observed at high [Ca^{2+}], in which Ca^{2+} (and Mg^{2+}) act reversibly at a low affinity inhibitory site (see Laver and Lamb, 1998 and review of such mechanisms in Lamb and Laver, 1998).

Thus, solution change experiments provide no support for adaptation behavior in individual RyRs. In cases where the P$_o$ does decline, it is due to a distinctive voltage- and P$_o$-dependent inactivation mechanism that cannot be reversed immediately by applying a larger Ca^{2+} stimulus (Laver and Lamb, 1998). Importantly, as the solution change experiments reveal that RyRs can inactivate in response to a sustained [Ca^{2+}] stimulus, it is imperative to show that an "adapted" RyR is responsive to a larger stimulus and not simply inactivated. This has only been documented in one case (Figure 3 in Györke and Fill, 1993).

The difficulties associated with distinguishing between adaptation and inactivation also are demonstrated by examining the model proposed for activation-adaptation by Zahradníková and colleagues (Figure 5 A in Zahradníková et al., 1999,) which is actually describing an inactivation process. In this model, the overall adaptation behavior arises because the RyR moves into a set of inactivated and low P$_o$ states from which it can exit only slowly. Consequently, once an in-

dividual RyR has adapted, it cannot be rapidly reactivated by a second rapid rise in [Ca^{2+}], which is the defining feature of adaptation (Györke and Fill, 1993). The model can describe only an adaptation-like response occurring in a population of RyRs, where some RyRs are activated by a first stimulus (and then inactivate) and the second stimulus activates RyRs that had not been activated by the first stimulus. An appropriate model must be able to account for how an individual RyR can be activated to a relatively high P$_o$ (>0.3) by a very small increase in [Ca^{2+}] (from 100 to 200 nM), adapt, and then be reactivated immediately by a further step increase in [Ca^{2+}]. Furthermore, any such model must be able to correctly describe the response of a RyR to the actual [Ca^{2+}] stimulus occurring in flash photolysis experiments (i.e., a Ca^{2+} spike–step stimulus). Specifically, it must predict a decline in P$_o$ with a single slow ($\sim$1 s) time constant, without any initial rapidly declining phase.

Problem of [Ca^{2+}] Measurements in the Flash Photolysis Experiments

A further problem relates to the determination of the free [Ca^{2+}] in the flash photolysis experiments. To demonstrate adaptation in a RyR, the [Ca^{2+}] must be rapidly changed from one known steady level to another higher level. In the flash photolysis studies, this was achieved by placing the laser light guide near the lipid bilayer containing the RyR to restrict diffusion, and then photolysing some of the nitrophen in the intervening region. The [Ca^{2+}] recordings for the photolysis experiments of Valdivia et al. (1995) show that the free [Ca^{2+}] was not steady after photolysis but dropped two- to threefold in 2 s (e.g., from 10 to 3 μM or 1 to 0.5 μM), raising the question of how much this contributed to the observed decrease in RyR activation. Furthermore, there seems to be a problem with the [Ca^{2+}] estimates associated with the adaptation experiment of Györke and Fill (1993; see their Figure 3), as it is not possible with Ca^{2+} buffering by nitrophen alone for two equal intensity laser flashes to raise the [Ca^{2+}] from an initial level of $\sim$100 nM to $\sim$220 nM and then to $\sim$500 nM. If the first flash more than doubled the free [Ca^{2+}], it would have had to destroy enough nitrophen so that (after Ca^{2+} rebinding) the free nitrophen dropped to less than half of its initial level (Lamb and Stephenson, 1995), and, consequently, after a second similar flash there would be effectively no free nitrophen and the free [Ca^{2+}] would be >5 μM, not $\sim$500 nM. This inconsistency can be explained by Ca^{2+} buffering by the Ca^{2+} ionophore resin used to measure the [Ca^{2+}]. Györke and colleagues estimated the [Ca^{2+}] changes caused by the photolysis procedure by removing the bilayer containing the RyR and painting a Ca^{2+} ionophore resin across the bilayer aperture (Györke

and Fill, 1993, 1994; Györke et al., 1994; Velez et al., 1997; Györke, 1999; Zahradníková et al., 1999). The steady-state response of this Ca^{2+} electrode was calibrated by changing the $[Ca^{2+}]$ in the whole of the cis bath (the light-guide side) and having a known (100 mM) Ca^{2+} solution on the other (trans) side (Györke et al., 1994). When the $[Ca^{2+}]$ was raised in the flash photolysis, however, the increase occurred in a very small volume ($\sim$0.1 µl), and the apparent free $[Ca^{2+}]$ reported by the resin may have been reduced because of Ca^{2+} binding to the Ca^{2+} ionophore in the resin matrix. Thus, when the response of the RyR was measured, with a lipid bilayer present instead of resin, the rise in free $[Ca^{2+}]$ may have been considerably higher than when the Ca^{2+} ionophore resin was present. In other words, the $[Ca^{2+}]$ measurements may have underestimated the Ca^{2+} stimulus applied to the RyR in the bilayer experiment. Any such underestimation of the $[Ca^{2+}]$ would mean also that the Ca^{2+} spike time integrals in Fig. 1 B are underestimates, though it would not change their rank order or our interpretation. In summary, the prolonged RyR activity occurring with intense flashes might be because of the Ca^{2+} spike causing initial rapid activation of the RyR and the relatively high steady $[Ca^{2+}]$ helping keep the channel activated for some time before eventually dissipating.

Studies of Ca^{2+} Release by RyRs In Situ

In cellular preparations, Ca^{2+} release in some circumstances decreases over time but can be increased again by applying a larger stimulus. This response of a population of RyRs in situ can occur by many mechanisms without there being any adaptation in individual RyRs (Lamb and Laver, 1998). The adaptive behavior observed at the cellular level can be divided in two general categories, in which the Ca^{2+} release declines over seconds to minutes or on a millisecond time scale (Lamb and Laver, 1998). When the role of adaptation in the former case was specifically examined, using caffeine-activated Ca^{2+} release in PC12 cells containing the cardiac RyR isoform, adaptation was ruled out and the decline of Ca^{2+} was shown to be due to alterations in the amount of Ca^{2+} in the intracellular stores (Koizumi et al., 1999). Similarly, the rapid termination of Ca^{2+} release in cardiac cells was found to be due to use-dependent inactivation of the RyRs and not to adaptation (Sham et al., 1998). There is no evidence for RyR adaptation from studies of Ca^{2+} release in situ. We also note that if adaptation, as described by Györke and Fill (1993), were to occur in vivo, the greatly heightened sensitivity of the RyRs to very small increments in $[Ca^{2+}]$ would hinder, rather than help, the ability of the cell to produce a graded response to submaximal stimuli, which is an important property of cardiac muscle.

In conclusion, we find no convincing evidence that individual RyRs show adaptation as originally defined by Györke and Fill (1993). That said, the response of RyRs to the Ca^{2+} spikes generated in the flash photolysis experiments may reflect important properties of the dynamic response of the RyR. As brief Ca^{2+} spike stimuli may occur in vivo, such behavior could be important in the generation of Ca^{2+} sparks (Lamb, 1997), though there remains the problem that none of the mechanisms of termination of Ca^{2+} release so far observed in isolated RyRs, be they called adaptation or inactivation, are rapid enough to explain events occurring in vivo (Lamb and Laver, 1998; Laver and Lamb, 1998). Clearly, many questions remain unanswered and we need more information about the responses of RyRs to sudden changes in $[Ca^{2+}]$, particularly from studies in which true Ca^{2+} steps can be applied so that the effects of the Ca^{2+} spike and step can be studied separately.

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