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Ca^{2+} Feedback on "Quantal" Ca^{2+} Release Involving Ryanodine Receptors

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SUMMARY

The influence of luminal and cytoplasmic Ca^{2+} on the ability of ryanodine-sensitive stores to undergo multiple partial ("quantal") releases has been assessed. Increased luminal Ca^{2+} levels do indeed modulate sarcoplasmic reticulum Ca^{2+} release by lowering the threshold agonist concentration required to elicit release, but the decrease in luminal Ca^{2+} that accompanies a partial release is not sufficient by itself to terminate release. Similarly, an increase in cytoplasmic Ca^{2+} lowers the threshold agonist concentration required to elicit release; thus, the bulk cytoplasmic Ca^{2+} levels attained during a release would only stimulate further release, not terminate it before it reached completion. Very high cytoplasmic Ca^{2+} levels (1–3 mM) also triggered release but were unable to terminate release before reaching completion. Thus, even the high local cytoplasmic Ca^{2+} concentration that might accompany release would also not terminate release. It is concluded that Ca^{2+} feedback can modulate release through ryanodine receptors but that it does not account for the properties of quantal release. The low affinity inhibitor tetracaine induces a decrease in the extent of release that cannot be explained solely by heterogeneous caffeine sensitivity of the stores. The results are interpreted in terms of a scheme that includes (i) heterogeneous sensitivity of stores, conferred in part by differences in luminal Ca^{2+} content and (ii) adaptive behavior on the part of individual ryanodine receptors.

The ability of ryanodine receptors to mediate multiple partial releases of Ca^{2+} from internal stores in response to step increases in agonist concentrations has been documented by several groups (1–3). This phenomenon bears similarity to the "quantal" release of Ca^{2+} from internal stores sensitive to $InsP_3$ (4). The mechanism or mechanisms responsible for these partial releases of Ca^{2+} remain highly controversial, with two main theories propounded. It has been proposed that receptors of heterogeneous agonist sensitivity are grouped together in isolated stores such that some stores release all their Ca^{2+} at low agonist concentration, and other stores follow suit only at higher agonist concentrations. In contrast to such static receptor sensitivity, it has been proposed that some other feedback mechanism regulates the agonist sensitivity of even individual release channel receptors. In its original form, this proposal involved luminal Ca^{2+} being the feedback system (5). Experimental demonstrations of such dynamic receptor sensitivity included the observations of a low level of steady state flux of Ca^{2+} from $InsP_3$-sensitive stores (6) or the dynamic adjustment of Ca^{2+} sensitivity of individual ryanodine receptors in planar lipid bilayers (7). The latter result was obtained under conditions in which luminal Ca^{2+} concentration was low and would not have varied.

In view of the postulated involvement of luminal Ca^{2+} in such dynamic sensitivity and the known sensitivity of both $InsP_3$ and ryanodine receptors to cytoplasmic Ca^{2+} concentration (8), we investigated further the role of Ca^{2+} in the quantal release of Ca^{2+} from internal stores involving ryanodine receptors. Our earlier study (3) showed that multiple releases take place regardless of whether released Ca^{2+} is resequestered, suggesting that Ca^{2+} redistributions do not control the partial release behavior. The results of the experiments reported here show that Ca^{2+} concentration changes on either side of the ryanodine receptor can modulate incremental Ca^{2+} release behavior, in particular by altering agonist sensitivity, but that these effects by themselves are unable to account for the stopping and restarting of release. Results with inhibitors provide additional strong evidence against multiple partial releases being due purely to true quantal behavior and in favor of a more dynamic contribution to incremental release behavior, one that does not rely primarily on luminal Ca^{2+} levels.

Materials and Methods

Heavy microsomes and purified terminal cisternae were prepared from rabbit skeletal muscle as described in Dettbarn *et al.* (3). Protein concentrations were determined using Coomassie Protein Assay Reagent (Pierce, Rockford, IL) using bovine serum albumin as a standard.

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ABBREVIATIONS: CICR, Ca^{2+}-induced Ca^{2+} release; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $InsP_3$, inositol-1,4,5-trisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

Spectrophotometric measurements of SR Ca^{2+} uptake and release were performed in a diode array spectrophotometer (HP 8451A; Hewlett Packard, Palo Alto, CA). The standard assay medium included 100 mM KCl, 20 mM K-MOPS, 2.5 mM potassium phosphate, 1 mM Mg-ATP, 5 mM Na_2 phosphocreatine, 20 μg/ml creatine phosphokinase, and 0.2 mM antipyrylazo III, pH 6.8. Some experiments (see Figs. 1–4) used no phosphate, with pH 6.95 medium at 37°. Extravesicular Ca^{2+} concentration changes were followed by measuring antipyrylazo III absorbance at A_{710}–A_{790}.

Certain ^{45}Ca efflux determinations were carried out in the same medium at the same temperature but with ^{45}Ca during the initial phase of preloading of endogenous Ca^{2+} associated with the sample. The additions of 1–3 mM Ca^{2+} were then made, followed by the addition of 5 mM EGTA, appropriately alkalinized to maintain pH of the final mixture. Efflux was monitored by measuring the Ca^{2+} remaining in the vesicles by first quenching the reaction through the addition of 100-μl aliquots to 0.9 ml of ice-cold 100 mM KCl, 20 mM MOPS, 1 mM EGTA, 10 mM $MgCl_2$, and 20 mg/liter ruthenium red, pH 6.8. At the conclusion of each run, the quenched 1-ml mixtures were filtered by conventional manifold filtration with Millipore HAWP filters (0.45-μm effective pore size) and washing with three 3-ml washes of ice-cold quench medium.

Other ^{45}Ca efflux determinations involved passive loading of purified terminal cisternae with ^{45}Ca. This was achieved by first sedimenting terminal cisternae (15 mg/ml) out of their storage medium containing 10% sucrose with a 5-min full-speed spin in an airfuge (Beckman Instruments, Palo Alto, CA); resuspension in sucrose-free 100 mM KCl and 20 mM MOPS, pH 6.8, with airfuging again to wash; and resuspension in its original volume of 100 mM KCl and 20 mM MOPS, pH 6.8. To this suspension, 5 mM Ca^{2+} spiked with ^{45}Ca was added, and the mixture was equilibrated overnight on ice. For efflux determinations, 10 μl of the mixture was diluted into 1 ml of 100 mM KCl, 20 mM MOPS, and 20 mM EGTA, pH 6.9 at room temperature. Three 100-μl aliquots were withdrawn at 10-sec intervals, and each was added to 0.9 ml of quench medium as described above, followed in some cases by the addition of 2–8 mM caffeine from a concentrated stock at t = 40 sec. Three additional 100-μl aliquots were then withdrawn and quenched at 10-sec intervals, followed in some cases by elevation of the caffeine concentration to 22 mM and withdrawal of three additional aliquots for quenching and subsequent filtration as described above. All isotope data were normalized with respect to the initial (t = 10 sec) time point in each series.

Results

Partial releases of SR Ca^{2+} in response to step changes of caffeine concentration are shown in Fig. 1. Because we prefer to reserve the term quantal to refer to one specific and as-yet-unproved explanation for the phenomenon, we will refer to such releases interchangeably as partial, incremental, or quantal throughout this text. In Fig. 1, multiple partial releases were obtained under conditions in which released Ca^{2+} was given time to be resequestered. Note that the first three additions of caffeine failed to generate any detectable release of Ca^{2+}, and a large release with a plateau was obtained only on the fifth caffeine addition. If the amount of Ca^{2+} preloaded into the vesicles is increased (Fig. 1, B and C), not only are the releases of Ca^{2+} larger in amplitude, but the caffeine concentration required to initially activate release is reduced. Thus, the threshold concentration of agonist (caffeine) required to elicit release is lowered by increased luminal Ca^{2+} content. Three additional sets of experiments were carried out with 6.25, 12.5, and 18.75 nmol of Ca^{2+} preloading. In all experiments with 6.25 nmol of Ca^{2+} preloading, a large release with a plateau was obtained only on the fifth caffeine addition, whereas large releases were always obtained with the third caffeine addition if 12.5 nmol of Ca^{2+} had been preloaded and on the second caffeine addition when 18.75 nmol of Ca^{2+} had been preloaded. These differences are highly significant as assessed using a Student's paired t test.

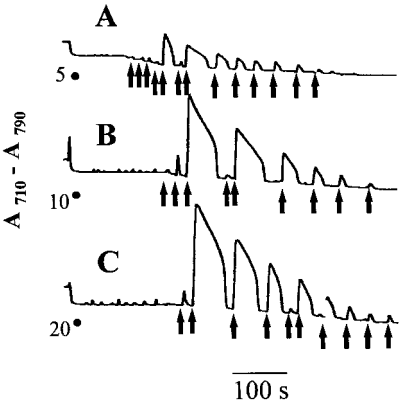

Fig. 1. Caffeine sensitivity of SR stores is affected by the luminal Ca^{2+} content. A, Multiple Ca^{2+} releases in response to step increases in caffeine concentration. Rabbit microsomes (600 μg) were preloaded with 6.25 nmol of Ca^{2+} in 1.25-nmol increments as described in the text. Subsequently, the vesicles were challenged with increasing concentrations of caffeine as indicated. Caffeine was never removed from the cuvette. B and C, Multiple Ca^{2+} releases in response to caffeine when the vesicles were loaded with more Ca^{2+} (12.5 nmol in B, 25 nmol in C). ●, 1.25-nmol Ca^{2+} additions. *Arrows*, 0.6 mM caffeine additions.

The results of the experiments in Fig. 1 were qualitatively consistent with the original Irvine hypothesis of luminal Ca^{2+} controlling quantal release, but they certainly did not prove the hypothesis. To more quantitatively assess the possible contribution that this change in sensitivity might make to subsequent incremental release, we determined how much of the Ca^{2+} taken up had been released by caffeine. If the Irvine hypothesis were to explain quantal release, then preloading the vesicles so that their luminal content would be the same as that experienced at the time of termination of release should result in a failure of the same caffeine concentration to elicit release.

This test was applied in Fig. 2. First heavy microsomes were loaded with 100 nmol of Ca^{2+} and challenged with 5 mM caffeine (Fig. 2A). This concentration of caffeine was chosen to give submaximal release (the release seen at 3 mM was just detectable). The response was a release of ~35 nmol of Ca^{2+}. We then preloaded the vesicles with progressively less Ca^{2+} (62.5, 37.5, or 12.5 nmol of Ca^{2+}) before challenging them with the same concentration of caffeine. As seen in Fig. 2, a release of Ca^{2+} was observed under all these conditions, contrary to the predictions of the Irvine hypothesis. Similar results were obtained with another sample that released less Ca^{2+} in response to 4 mM caffeine (not shown) and with purified terminal cisternae that released a greater proportion of the Ca^{2+} taken up (not shown). Because vesicles with even less Ca^{2+} in them than after a release remained responsive to the same submaximal concentration of caffeine, any decrease in caffeine sensitivity caused by a release did not seem to be sufficient by itself to account for the termination of release.

A separate test was made to determine whether heterogeneously loaded stores could account for multiple partial releases of Ca^{2+} in response to incremental caffeine additions.

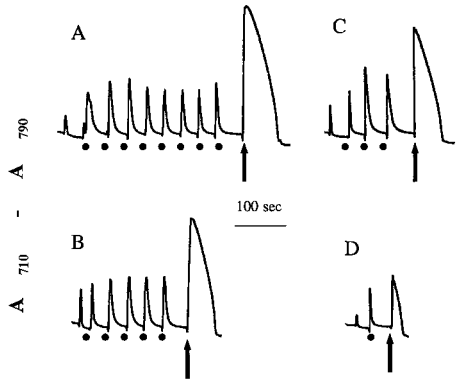

Fig. 2. The decrease in luminal Ca^{2+} caused by a submaximal caffeine-induced Ca^{2+} release cannot account for release termination. A, Heavy microsomes (380 μg) were preloaded with eight 12.5-nmol increments of Ca^{2+} (100 nmol total) and then challenged with 5 mM caffeine, releasing ~35 nmol Ca^{2+}. B–D, Heavy microsomes (380 μg) were preloaded with only 62.5 nmol (B), 37.5 nmol (C), or 12.5 nmol (D) of Ca^{2+} and then challenged with 5 mM caffeine. Release still took place. ●, 12.5-nmol Ca^{2+} additions. *Arrows*, 5 mM caffeine additions. *Traces* are representative of experiments performed in triplicate under each condition.

To perform this test, purified terminal cisternae were passively equilibrated in 5 mM Ca^{2+}/^{45}Ca-containing solutions to ensure that all vesicles were uniformly loaded with Ca^{2+}. Then, the vesicles were challenged with two steps of increasing concentration of caffeine. Although the threshold concentration of caffeine required to activate release may differ in the absence of ATP, the experiment diagrammed in Fig. 3 demonstrates that multiple responses are obtained. Similar results were obtained using 2 or 5 mM caffeine for the first caffeine challenge (not shown). Thus, the multiple responses to caffeine cannot be accounted for by populations of vesicles that are differentially loaded with Ca^{2+}.

However, because the effect of luminal Ca^{2+} was at least qualitatively consistent with the Irvine hypothesis, it remained possible that some other feedback system acting in concert with the luminal Ca^{2+} effect could result in release termination. The most obvious candidate to explore for such a contribution is the extravesicular (cytoplasmic) Ca^{2+} concentration because it increases whenever a release is taking place.

Accordingly, we preloaded vesicles to the same extent with Ca^{2+} and then exposed them to Ca^{2+}. Finally, the vesicles were challenged with step increases in caffeine concentration. If Ca^{2+} was administered in a fashion that it was not able to be appreciably sequestered before caffeine addition, then the threshold caffeine concentration required to activate release was lowered. Vesicles unresponsive to 1 mM caffeine at ambient resting Ca^{2+} concentration became responsive if Ca^{2+} was elevated just before caffeine addition (Fig. 4). This result was not surprising in that it probably represents a corollary to the much earlier observation that caffeine enhances Ca^{2+} sensitivity (9). In fact, it is clear from Fig. 4A that caffeine administered before Ca^{2+} addition led to a larger absorbance increase from the Ca^{2+} addition, as if some Ca^{2+}-induced Ca^{2+} release had indeed occurred. This result was not in agreement with a contributory role for elevated bulk cytoplasmic Ca^{2+} in terminating release. However, high local Ca^{2+} concentrations around the mouth of a conducting release channel might be more prone to induce inactivation of that channel because such local Ca^{2+} concentrations could easily exceed those of the Ca^{2+} concentrations administered in Fig. 4.

If the vesicles were exposed to only a 2–4-fold higher extravesicular Ca^{2+} concentration, another effect was encountered. Releases evoked by even high caffeine concentrations were slowed and attenuated (Fig. 5). These were Ca^{2+} concentrations only slightly higher than those achieved at the peak of most releases (15–20 μM total Ca^{2+}, which we calculate to be equivalent to 5–7 μM free Ca^{2+} under our experimental conditions at pH 6.8). The downward-going artifacts

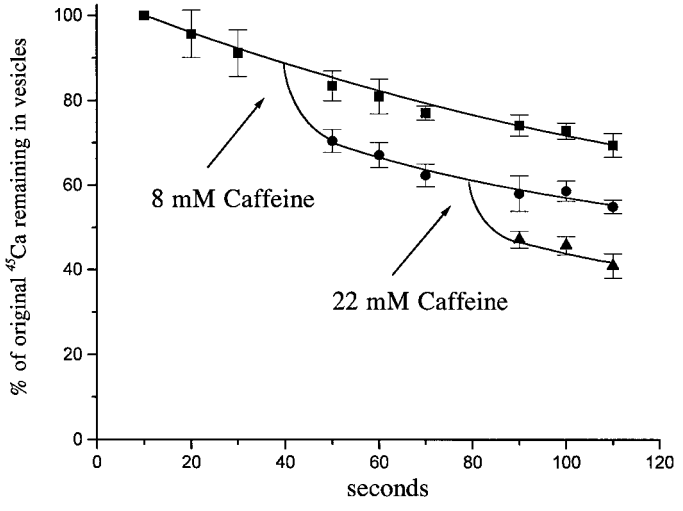

Fig. 3. Homogeneously loaded SR vesicles still display multiple partial releases of Ca^{2+}. Terminal cisternae at ~15 mg/ml were equilibrated overnight with 5 mM Ca^{2+}/^{45}Ca-containing solution as detailed in the text. After 100-fold dilution into EGTA-containing solution (■), in some cases the vesicles were challenged first with 8 mM caffeine (●); in some of these experiments, the caffeine concentration was raised to 22 mM (▲) 40 sec later. All experiments were performed at least in triplicate. *Error bars*, mean ± standard deviation. All *data points* at the same time point are statistically different from one another as assessed with a Student's paired *t* test ($p < 0.05$). *Curves* were drawn by eye.

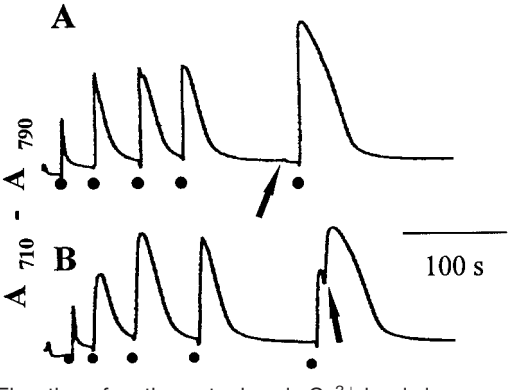

Fig. 4. Elevation of resting cytoplasmic Ca^{2+} levels increases agonist sensitivity. A, Heavy microsomes (380 μg) preloaded with 50 nmol of Ca^{2+} were then treated with 12.5 nmol of Ca^{2+} just before challenge with 1 mM caffeine. B, Microsomes were treated in the same way except that the caffeine was added before the last Ca^{2+} addition. ●, 12.5 nmol Ca^{2+} additions. *Arrows*, 1 mM caffeine additions. *Traces* are representative of experiments performed at least in triplicate, including (B) experiments (not shown) in which 62.5 nmol of Ca^{2+} was preloaded before caffeine addition to compensate for any additional Ca^{2+} taken up (in A) before caffeine addition. Regardless of whether 50 or 62.5 nmol of Ca^{2+} had been preloaded, caffeine still failed to elicit release unless the extravesicular Ca^{2+} was elevated.

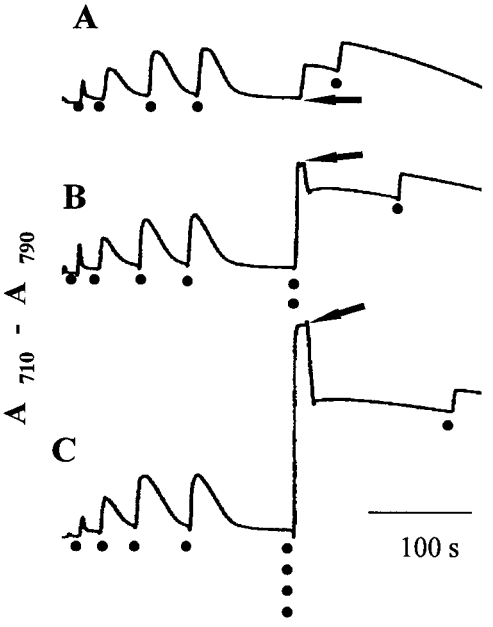

Fig. 5. Further elevation of resting cytoplasmic Ca^{2+} levels decreases subsequent caffeine-induced Ca^{2+} release. Heavy microsomes (380 μg) preloaded with 50 nmol of Ca^{2+} were then treated with 0, 25, or 50 nmol of Ca^{2+} just before the addition of 10 mM caffeine. Downward shifts in traces on caffeine addition are due to caffeine reduction of antipyrylazo III sensitivity to Ca^{2+}. ●, 12.5-nmol Ca^{2+} additions. *Arrows*, 10 mM caffeine additions. Due to its interaction with antipyrylazo III, caffeine induces a downward deflection in traces that is larger in the presence of high Ca^{2+}. *Traces* are representative of at least three determinations under each condition.

observed on caffeine additions in the presence of elevated Ca^{2+} might have partially obscured some remaining release.

To rule out further release by caffeine under such circumstances and to determine whether this inhibitory effect was due to CICR depletion of the vesicles or to an inactivating effect of the elevated extravesicular Ca^{2+}, equivalent experiments were performed with purified terminal cisternae using the Ca^{2+} ionophore A23187 to assess the Ca^{2+} remaining in the SR after the different treatments. As seen in Fig. 6, A and B, caffeine application reduced the A23187-releasable fraction considerably, but so did before Ca^{2+} addition (Fig. 6C). Much less Ca^{2+} was left to be released by A23187 subsequent to the caffeine (4.10 ± 0.31 nmol) or Ca^{2+} addition in Fig. 6C (4.52 ± 0.67 nmol versus control 33.69 ± 0.05 nmol), and thus substantial CICR had occurred in response to the Ca^{2+} addition, probably leading to depletion rather than inactivation.

In principle, high local Ca^{2+} concentration, which only occurs while a release channel is still conducting, could, together with a decrease in luminal Ca^{2+}, lead to release termination. When the release terminates, the high local Ca^{2+} concentration would dissipate very rapidly, and the system might then soon be susceptible to reactivation easily because the decrease in luminal Ca^{2+} alone had not been sufficient to prevent reactivation. To determine whether any Ca^{2+}-induced inactivation of release had also occurred, we determined whether high Ca^{2+} administered exogenously would lead to inactivation before depletion. As seen in Fig. 7, Ca^{2+} elevations of even 3 mM failed to terminate release of ^{45}Ca before completion, and subsequent addition of EGTA (to reduce the cytoplasmic Ca^{2+} to a level where no inactivation would take place) failed to reactivate release because little Ca^{2+} remained in the SR. Similar results were obtained with 1 mM Ca^{2+} (not shown).

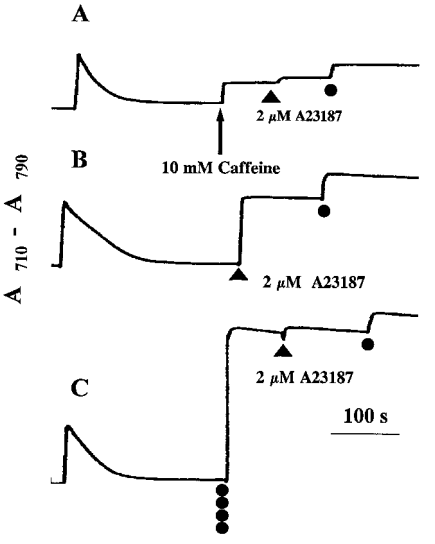

Fig. 6. CICR takes place even when vesicles are challenged with high cytoplasmic Ca^{2+}. Purified terminal cisternae (300 μg) added at the first upward deflection were allowed to take up endogenous Ca^{2+} associated with the sample and assay solution and then challenged with 10 mM caffeine (A), 2 μM A23187 (B), or 50 nmol of Ca^{2+} (C). At the end of each trace, 2 μM A23187 was added, followed by 12.5 nmol of Ca^{2+}. ●, 12.5 nmol Ca^{2+} additions. *Arrow*, 10 mM caffeine additions. *Arrowheads*, 2 μM A23187 additions. Experiments shown are representative of at least three separate determinations under each condition.

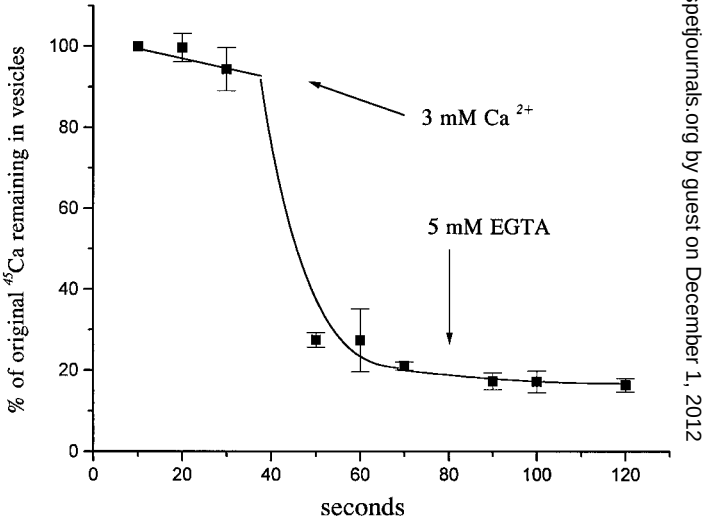

Fig. 7. Effect of high Ca^{2+} and its subsequent reduction on efflux of ^{45}Ca. Terminal cisternae (300 μg) were actively preloaded with endogenous Ca^{2+} spiked with ^{45}Ca in the presence of ATP. Aliquots were removed for Millipore filtration as described in the text. After three base-line readings, terminal cisternae treated as above were exposed to 3 mM total Ca^{2+} (2 mM free Ca^{2+}) at t = 40 sec. Subsequent addition of EGTA sufficient to lower the free Ca^{2+} concentration to 1.5 μM at t = 80 sec failed to reactivate release. The experiment was performed four times with results expressed as mean ± standard deviation. *Curve* was drawn by eye.

The results thus far suggest that neither luminal nor cytoplasmic Ca^{2+} changes can account for multiple partial releases. Because Irvine's luminal Ca^{2+} regulation hypothesis represented the original alternative to true quantal behavior,

does our dismissal of the luminal Ca^{2+} explanation represent indirect evidence in favor of true quantal behavior? To test this premise directly, we performed an additional set of experiments.

Microsomes were loaded with Ca^{2+} and then challenged with caffeine or caffeine in the presence of concentrations of tetracaine that only partially inhibited release. Two different concentrations of caffeine were used: one (4 mM) that elicited a clearly submaximal rate of release, and one (10 mM) that exhibited substantially larger, more prolonged release with a faster rate of release. A peculiarity of the tetracaine inhibition of release seen in Fig. 8 was that the dose-response curve (not shown) for tetracaine was unusually steep and clearly depended on the caffeine concentration. True quantal behavior would predict that the same vesicles that released (completely) in the presence of caffeine alone would also release (completely) in the presence of the low affinity antagonist tetracaine and that the amount released should be the same in the presence of tetracaine, even if the rate of release was reduced. This behavior was not observed experimentally. Instead, the amount of Ca^{2+} released seemed to be decreased as much as the rate of release (Fig. 8). These results suggest that some time-dependent process (either inactivation or adaptation) must have caused the release to terminate. Furthermore, it is unlikely that release termination was dependent on the length of time that conducting release channels were exposed to high local Ca^{2+} during release. Had this been the case, a 50% inhibition in release rate would have dictated a doubling of time necessary for any time- and Ca^{2+}-dependent process to take place, whereas lower release rates (4 versus 10 mM caffeine or tetracaine versus controls) were associated with more rapid cessation of release.

Discussion

The role of luminal Ca^{2+}. Our result that termination of release occurs under Ca^{2+} loading conditions where the same stimulus can still elicit release (Fig. 2) leads us to conclude that luminal Ca^{2+} by itself does not control quantal Ca^{2+} release of RyRs, as in the original Irvine $InsP_3$ receptor hypothesis. However, as suggested in that hypothesis, a decrease in luminal Ca^{2+} does provide a negative feedback to modulate release by increasing the threshold concentration of agonist required to elicit release. We used the concept of altered threshold to describe our results because they do not strictly discriminate between a change in affinity (sensitivity) of the RyR from a decreased cooperativity of binding. For the sake of convenience in the Discussion, we use the term "sensitivity" in its most general sense, to include either possibility. Our results are consistent with the positive feedback of increased intraluminal Ca^{2+} noted with isolated SR vesicles (10). Results obtained with ryanodine receptors studied in planar lipid bilayers with high intraluminal Ca^{2+} have been more varied. In the absence of ATP, high intraluminal Ca^{2+} has been reported to decrease conductance (11) or to have no effect (12), but in the more physiological presence of ATP, high intraluminal Ca^{2+} has a stimulatory effect on P_o (12), which would be in accord with our vesicular results, also obtained in the presence of ATP. Finally, the caffeine sensitivity of Fura-2-loaded adrenal chromaffin cells was enhanced by greater luminal Ca^{2+} content (1). Thus, a decrease in luminal Ca^{2+} is not the controlling factor that terminates release for ryanodine receptors.

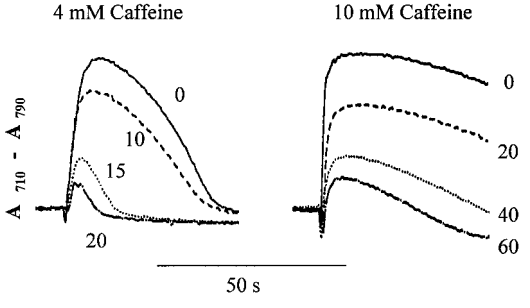

Fig. 8. Effect of tetracaine on caffeine-induced Ca^{2+} release. Heavy microsomes (380 μg) were preloaded with 100 nmol of Ca^{2+} in eight 12.5-nmol increments and challenged with 4 (*left*) or 10 (*right*) mM caffeine. *Top*, absence of tetracaine. Experiments were then repeated with 10, 15, and 20 μM tetracaine (*left*; concentrations as indicated) or 20, 40, and 60 μM tetracaine (*right*; concentrations as indicated). Traces in the presence of tetracaine demonstrated not only reduced rates of release of calcium but also reduced amounts of Ca^{2+} release. The control release with 4 mM caffeine represented 10 nmol of Ca^{2+}; that elicited by 10 mM caffeine was 13.5 nmol of Ca^{2+}. The tetracaine-induced decreases in release amplitudes shown are representative of at least three determinations of each set of conditions. With different samples, the concentration of tetracaine needed to achieve these effects differed slightly, but tetracaine always reduced release amplitudes, and more tetracaine was required to inhibit release at higher caffeine concentrations.

Experimental evidence suggests no greater role for luminal Ca^{2+} in regulating $InsP_3$-induced Ca^{2+} release. Thus, Hannaert-Merah *et al.* (13) reported no effect of luminal Ca^{2+} on $InsP_3$ bound, and there have been contradictory reports regarding whether partial Ca^{2+} depletion reduced the $InsP_3$ sensitivity of release (14, 15). Furthermore, Ferris *et al.* (16) clearly found termination of rapid $InsP_3$-mediated flux even as luminal Ca^{2+} was likely to be increasing.

The role of cytoplasmic Ca^{2+} elevations. Previously, we noted that multiple releases were obtained from SR regardless of whether extravesicular Ca^{2+} was allowed to remain high and concluded that it was unlikely that extravesicular (cytoplasmic) Ca^{2+} plays a significant role in controlling release (3). This conclusion still holds for bulk cytoplasmic Ca^{2+} levels. The experiments presented here suggest that even local cytoplasmic Ca^{2+} concentrations in the vicinity of a conducting release channel play no greater role. Although elevated local Ca^{2+} in the vicinity of a reconstituted single channel can inactivate it (11, 17, 18), our results suggest that any inactivation taking place in our vesicle studies is too incomplete to terminate release before vesicle depletion (Fig. 7). Our results with tetracaine and different caffeine concentrations also argue against negative feedback by released Ca^{2+}: if release did inactivate or adapt before completion, the inactivation would have to have been independent of Ca^{2+} flux through the channel because the reduction in flux by tetracaine or lower caffeine did not obviously slow inactivation. Thus, local Ca^{2+} concentration changes by themselves also cannot account for multiple partial releases. As a consequence, our results do not support application to the RyR of the model of Swillens *et al.* (19), which suggests that Ca^{2+} conduction through a release channel is the critical factor in terminating release.

The adaptation reported by Gyorke and Fill (7) was observed in the absence of luminal Ca^{2+} or Ca^{2+} conducted through the channel and in the presence of maintained mod-

est elevations in cytoplasmic Ca^{2+}. Thus, single-channel studies suggest that Ca^{2+} concentration changes on either side of the membrane do not account for multiple bursts of channel activity. Our conclusions from this more macroscopic study of channel behavior are in agreement. Gyorke and Fill (7) have been challenged on the basis that the Ca^{2+} elevations they produced might have included rapid spikes to much higher levels than they measured (20). Nevertheless, because channel deactivation is very rapid (17, 21), these rapid spikes do not seem to account for the much slower reduction of channel activity attributed to adaptation (21). Some experiments involving rapid solution changes have been reported with results displaying similar slow reduction in channel activity after Ca^{2+} concentration increases (17, 22). Adaptation might be slower in single-channel studies than *in situ* (24) because Cs^+ or K^+ was used as the current carrier rather than Ca^{2+}, and the Ca^{2+} levels achieved at the inactivating site might have been considerably smaller. It is well known that many skeletal RyRs exhibit decreased open probability when Ca^{2+} is elevated in the 10 μM-1 mM concentration range (8, 11), an effect manifested in vesicular studies as a diminished rate of release (23), and Tripathy and Meissner (18) concluded that Ca^{2+} conducted through the channel could contribute to inactivation. Low affinity Ca^{2+}-dependent inactivation of release might still provide a mechanism for enhancing the rate of adaptation to a maintained stimulus.

With regard to $InsP_3$ receptors, negative feedback of released Ca^{2+} has either not been noted (25) or when noted (26) was unmasked at such low concentrations of chelator that it could have been a pharmacological side effect of the chelator (see Ref. 27) rather than true buffering of Ca^{2+} at the mouth of a conducting channel. Inactivation of the $InsP_3$ receptor by Ca^{2+} and by $InsP_3$ has also been reported (28), although as in the current study, Parys *et al.* (29) did not consider it responsible for the quantal release they observed.

Adaptation, true quantal behavior, or both?. True quantal release in which discrete stores with different static sensitivities individually release all of their Ca^{2+} or none at all would not account by itself for our results with tetracaine because in that case the individual stores that were sensitive to caffeine should have continued to release all their contents, albeit at a reduced rate. Furthermore, we provided previously evidence with isolated SR that caffeine can release some Ca^{2+} and that when the caffeine is diluted and its concentration subsequently restored, further release is obtained (3), another argument against true quantal release. Both arguments would apply to any static sensitivity explanation for incremental release, whether it involved different sensitivities due to differences in luminal load (current study), heterotetrameric release channels (30, 31), or different release channel densities (32).

Nevertheless, differential release from discrete $InsP_3$-sensitive stores has been documented in several more intact preparations (33–35) in which stores might not be continuous. In addition, Cheek *et al.* (1, 2) provided strong evidence in favor of true quantal release from caffeine sensitive stores in adrenal chromaffin cells using ryanodine to show that low caffeine concentrations activated only some channels (1). Cheek *et al.* (2) further determined that depletion of stores sensitive to low caffeine (achieved by multiple challenges with low caffeine in the presence of ryanodine or in the absence of Ca^{2+} in the medium) still revealed a large Ca^{2+} store sensitive to higher caffeine concentration. These results argue in favor of a heterogeneously sensitive population of stores (true quantal release) for their cells.

True quantal release and adaptation need not be mutually exclusive (36). Indeed, our results and those of Lukyanenko *et al.* (39) *in situ* indicate that discrete caffeine-sensitive stores could also become heterogeneously sensitive if they were loaded with Ca^{2+} to different extents. Even though adaptive behavior has been clearly shown in cardiac myocytes (24), highly localized releases (sparks) have also been described in myocytes (37), and these would be more consistent with release from discrete independently operated stores, as in true quantal release. Although stores seem discrete in skeletal myotubes, they exhibit heterogeneity in their caffeine sensitivity in a fashion that nevertheless indicates the presence of adaptation (38).

RyRs displaying adaptation in a population of heterogeneously sensitive stores could also explain three experimental results previously mentioned,which have been interpreted in favor of one interpretation or the other exclusively. First, if two successive challenges with the same submaximal caffeine concentration were administered with insufficient time allowed in-between for store reloading (3), heterogeneously sensitive stores with adapting RyRs would respond with some vesicles releasing some of their calcium on the first challenge, and then the second challenge would result in additional release, as observed (3), because the same vesicles released more calcium. Second, if a submaximal caffeine concentration elicited partial release, the effect of a low affinity inhibitor like tetracaine on heterogeneously sensitive stores with adapting RyRs would be to have the same vesicles releasing calcium at a lower rate for the same period of time, with the final result of a release of a smaller amount of calcium, as observed here (Fig. 8). In the third case, if heterogeneously sensitive stores with adapting RyRs were exposed to multiple challenges with the same caffeine concentration under conditions that they were unable to reload (2), the first challenge would result in some stores releasing some of their calcium. With subsequent challenges the same stores would release more of their calcium until they became depleted, but other less sensitive stores would retain their calcium until a much higher caffeine concentration was applied, as observed (2).

These arguments raise the spector that several different processes may together result in the unique phenomenon of multiple partial releases of Ca^{2+} stores in response to incrementally increasing agonist concentrations. If heterogeneous sensitivity can be conferred by luminal Ca^{2+}, as our results indicate, then the density of pump sites in individual stores could be an important determinant of store sensitivity. Whatever mechanism accounts for the adaptive contribution to quantal release, it will have to abide by the thermodynamic constraints identified by Stern (40). Regardless of the mechanisms that cause it, the incremental nature of this quantal release undoubtedly also contributes to the graded nature of releases involving both the RyR and the $InsP_3$ receptor, systems that would otherwise be expected to become all-or-none due to the positive feedback of released Ca^{2+} on further release.

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