Quantal Ca^{2+} mobilization by ryanodine receptors is due to all-or-none release from functionally discrete intracellular stores

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Low caffeine concentrations were unable to completely release the caffeine- and ryanodine-sensitive intracellular Ca^{2+} pool in intact adrenal chromaffin cells. This 'quantal' Ca^{2+} release is the same as that previously observed with inositol Ins(1,4,5)P_3-induced Ca^{2+} release. The molecular mechanism underlying quantal Ca^{2+} release from the ryanodine receptor was investigated using fura-2 imaging of single chromaffin cells. Our data indicate that the intracellular caffeine-sensitive Ca^{2+} pool is composed of functionally discrete stores, that possess heterogeneous sensitivities to caffeine. These stores are mobilized by caffeine in a concentration-dependent fashion, and, when stimulated, individual stores release their Ca^{2+} in an 'all-or-none' manner. Such quantal Ca^{2+} release may be responsible for graded Ca^{2+} responses in single cells.

INTRODUCTION

The release of Ca^{2+} from intracellular stores is the trigger for a diverse range of cellular activities including muscle contraction, secretion and enzyme activation. This internal mobilization of Ca^{2+} is mediated either by ryanodine receptors (RyR) (Lipscombe et al., 1988; McPherson et al., 1991; Friel and Tsien, 1992; Henzi and MacDermott, 1992), or by *myo*-inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3R). Both receptor types are similar in that they may be regulated by luminal Ca^{2+} (Nelson and Nelson, 1990; Missiaen et al., 1991, 1992; Parys et al., 1993; Cheng et al., 1993), phosphorylation (Witcher et al., 1991; Burgess et al., 1991; Wang and Best, 1992), adenine nucleotides (Lai et al., 1988; Maeda et al., 1991; Oyamada et al., 1993) and cytoplasmic Ca^{2+} (Bezprozvanny et al., 1991). The latter regulator is particularly important as it indicates that both receptors operate as Ca^{2+}-induced Ca^{2+} release (CICR) channels, although IP_3R requires the simultaneous presence of IP_3 (Berridge, 1993).

An additional functional similarity between RyR and IP_3R is that both receptors have been shown to release Ca^{2+} in a quantal manner. In its simplest form, quantal Ca^{2+} release denotes the inability of sub-maximal concentrations of caffeine or IP_3 to completely empty the caffeine-sensitive or IP_3-sensitive store respectively [reviewed by Taylor and Richardson (1991) and Bootman (1994)]. This effect has been observed using unidirectional cation flux measurements, confirming that it does not simply result from a steady state where release and reuptake are balanced. Quantal Ca^{2+} release from IP_3R has been described in both permeabilized (Muallem et al., 1989; Taylor and Potter, 1990; Meyer and Stryer, 1990; Renard-Rooney et al., 1993; Short et al., 1993) and intact (Parker and Ivorra, 1990, 1993; Bootman *et al.*, 1992) cells, and from RyR in intact cells (Cheek et al., 1993a). Furthermore, confocal imaging experiments have suggested that the fundamental quantal units may well be individual IP_3R or RyR molecules (Parker and Yao, 1991; Cheng et al., 1993), producing discrete, highly localized Ca^{2+} elevations.

Quantal Ca^{2+} release represents an elementary event in stimulus–response coupling, but the molecular mechanisms that underlie this release process are unknown. From experiments investigating IP_3-mediated Ca^{2+} release, two mechanisms have been proposed [reviewed by Taylor and Richardson (1991) and Bootman (1994)]. Firstly, it was suggested that Ca^{2+} is released in an all-or-none manner from functionally discrete intracellular stores that have a heterogeneous sensitivity to IP_3 (Parker and Ivorra, 1990, 1993; Parker and Yao, 1991; Oldershaw et al., 1991; Ferris et al., 1992). The second model proposed that Ca^{2+} stores are homogeneously sensitive to IP_3 but the IP_3-induced Ca^{2+} release is attenuated by the concomitant decrease of the luminal Ca^{2+} concentration (Irvine, 1990; Tregear et al., 1991; Missiaen et al., 1991, 1992; Parys et al., 1993; Loomis-Husselbee and Dawson, 1993). A further mechanism was recently proposed on the basis of experiments showing that RyR can adapt to prolonged Ca^{2+} stimulation (Györke and Fill, 1993); a rapid, sustained increase in cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) evoked only a transient phase of Ca^{2+}-channel activity, even though the RyR retained the ability to respond to an additional $[Ca^{2+}]_i$ increase (Györke and Fill, 1993, 1994; but see Lamb et al., 1994). This would be analogous to the concept of increment detection, as previously suggested for the IP_3R (Meyer and Stryer, 1990).

To investigate the mechanism underlying quantal Ca^{2+} release from RyR in single adrenal chromaffin cells, we developed an experimental protocol involving repetitive applications of the RyR agonist caffeine, given in the presence or absence of ryanodine. Ryanodine can lock activated RyR in their open configuration, and thus provide a 'memory' of those receptors that have been activated. Our experiments reveal that RyR adaptation is not responsible for quantal Ca^{2+} release from caffeine-sensitive Ca^{2+} stores. Also, we conclude that RyR have heterogeneous sensitivities to caffeine, and that when stimulated, RyR release Ca^{2+} from functionally discrete intracellular stores in an all-or-none manner. Such a release mechanism may underlie the ability of the whole cell Ca^{2+} response to be graded.

Abbreviations used: $[Ca^{2+}]_i$, cytoplasmic $[Ca^{2+}]$; $[Ca^{2+}]_o$, extracellular $[Ca^{2+}]$; CICR, Ca^{2+}-induced Ca^{2+} release; DMEM, Dulbecco's modified Eagle's medium; fura-2 AM, fura-2 acetoxymethyl ester; IP_3, *myo*-inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptors; RyR, ryanodine receptors.

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MATERIALS AND METHODS

Materials

Fura-2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR, U.S.A.), ryanodine from ICN Biomedicals (High Wycombe, Bucks., U.K.) and cell culture materials from Gibco (Paisley, Scotland, U.K.). All other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Isolation and culture of chromaffin cells

Chromaffin cells were dissociated from bovine adrenal medullas as described previously (Cheek et al., 1993a,b) and resuspended in Ca^{2+}-free Krebs–Ringer buffer comprising 145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose and 20 mM Hepes, pH 7.4 (buffer A). For primary tissue culture, cells were washed in buffer A and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM Hepes, 10% (v/v) foetal calf serum, 8 μM fluorodeoxyuridine, 50 μg/ml gentamycin, 10 μM cytosine arabinoside, 2.5 μg/ml fungizone, 25 i.u./ml penicillin and 25 μg/ml streptomycin (O'Sullivan et al., 1989). The cells were purified by differential plating for 2 h, after which time the non-adherent chromaffin cells were re-suspended in fresh DMEM, plated onto 22 mm diameter glass coverslips at a density of 1×10^5 cells/ml in 3 ml of medium and placed in a humidified atmosphere (CO_2/air, 1:19) at 37 °C. Cells were used 2–5 days after isolation.

Fura-2 measurements of single cells

Cells cultured on glass coverslips (see above) were washed with buffer A containing 3 mM $CaCl_2$, and loaded with fura-2 by incubation with 2 μM fura-2 AM for 35 min at room temperature (20 °C) in the presence of 0.05% (v/v) Pluronic F-127 (BASF Wyandotte). Cells were then washed by incubation for a further 45 min in buffer alone, and then imaged at 37 °C as previously described (Cheek et al., 1993a,b). A coverslip was mounted at 37 °C on the stage of a Nikon diaphot, inverted epifluorescence microscope. Fluorescent images were obtained by alternate excitation at 340 and 380 nm (40 ms each wavelength) using twin xenon arc lamps (Spex Industries Inc, Edison, NJ, U.S.A.). The emission signal at 510 nm was collected by a charge-coupled device intensifying camera (Photonic Science, Robertsbridge, U.K.) and the digitized signals were stored and processed using an Imagine image-processing system (Synoptics Ltd., Cambridge, U.K.) as described previously (O'Sullivan et al., 1989; Moreton, 1991; Cheek et al., 1993a,b). The fluorescence ratio was obtained at video rate and filtered with a time constant of 200 ms.

Formation of the ratio image was implemented in a look-up table, computed from the formula given by Grynkiewicz et al. (1985):

$$[Ca^{2+}]_i = K_d \frac{R - R_{min.}}{R_{max.} - R} \cdot \frac{S_{f2}}{S_{b2}}$$

where K_d is the dissociation constant for fura-2 and Ca^{2+} (224 nM), R is the intensity ratio for fluorescence at the two chosen wavelengths, $R_{min.}$ and $R_{max.}$ are ratios at zero and saturating $[Ca^{2+}]_i$ respectively, and S_{f2}/S_{b2} is the ratio of excitation efficiencies for free and bound fura-2 at the higher of the two wavelengths. All ratios were determined empirically using the *in vitro* calibration method (Moreton, 1992) by measuring the fluorescence intensities of bulk solutions of fura-2 free acid in $CaCl_2$/EGTA buffers prepared in an intracellular medium. Recorded video data were played back through Imagine, using a different program, to re-digitize into a frame-store. Data were sampled at 2 s intervals.

A nominally Ca^{2+}-free medium was obtained by adding 4 mM EGTA to cells in buffer A containing 3 mM $CaCl_2$. This was calculated to give a free $[Ca^{2+}]$ of < 2 μM (Maxchelator; Dr. C. Patton, Stanford University, Stanford, CA, U.S.A.). In this study, a response was defined as the elevation of $[Ca^{2+}]_i$ to > 10 nM. As the average resting $[Ca^{2+}]_i$ in the chromaffin cells was 22 ± 1 nM (mean $\pm$ S.D., $n = 114$), this represented a significant $[Ca^{2+}]_i$ elevation.

Use of caffeine and ryanodine to deplete the intracellular Ca^{2+} store

The effect of ryanodine and caffeine in inhibiting the putative CICR channel (see Figure 3e below) was achieved by incubating fura-2-loaded single cells with 10 μM ryanodine (stock 100 mM in methanol), and then adding a maximal concentration of caffeine (40 mM; Cheek et al., 1993a) to activate the channel in 30 s pulses over a 20 min period. At this concentration, ryanodine is expected to bind to activated RyR and lock them into an open sub-conductance state, thereby preventing the Ca^{2+} store from refilling (Stauderman and Murawsky, 1991; Buck et al., 1992; Cheek et al., 1993a). This notion is supported by the observation that the third pulse of 40 mM caffeine had no significant effect on $[Ca^{2+}]_i$ (Figure 3e), suggesting that the entire Ca^{2+} pool had been depleted by ryanodine after application of the first two caffeine pulses.

RESULTS AND DISCUSSION

Using populations of fura-2-loaded chromaffin cells, we have previously shown that maximal release of intracellularly stored Ca^{2+} occurs at 40 mM caffeine, with an EC_{50} of 12 mM caffeine, and is inhibited in a use-dependent fashion by 1 or 10 μM ryanodine (Cheek et al., 1993a). These data confirm that caffeine activates RyR in chromaffin cells (Stauderman and Murawsky, 1991) to mobilize Ca^{2+} from intracellular stores. The release mechanism was found to be quantal in nature: sub-maximal caffeine concentrations were unable to completely deplete the caffeine-sensitive store (Cheek et al., 1993a). The mechanism underlying the quantal response is unknown, but from experiments investigating IP_3-mediated Ca^{2+} release (Taylor and Richardson, 1991) two schemes can be proposed (Figure 1). These involve either partial Ca^{2+} release from all the stores (Figure 1a) or all-or-none Ca^{2+} release from functionally discrete stores (Figure 1b). In the present study, we have tried to distinguish between these two possibilities and have concluded that caffeine-induced Ca^{2+} release in single chromaffin cells depends upon an all-or-none release mechanism, as depicted in Figure 1(b).

Quantal Ca^{2+} release from RyR in single intact adrenal chromaffin cells was demonstrated using a sustained perfusion of caffeine in nominally Ca^{2+}-free medium (Figure 2). The data show that 3 mM caffeine [a sub-maximal concentration (Cheek et al., 1993a)] released only a fraction of the caffeine-sensitive stores in these cells; for each cell, irrespective of the extent of response to 3 mM caffeine, application of 40 mM caffeine almost always evoked an additional Ca^{2+} release. This pattern of response was unaffected by the length of the period between application of 3 mM and 40 mM caffeine (Figures 2a–2c), suggesting that the response was not attributable to a rapid release phase followed by a reduced rate of release. A similar

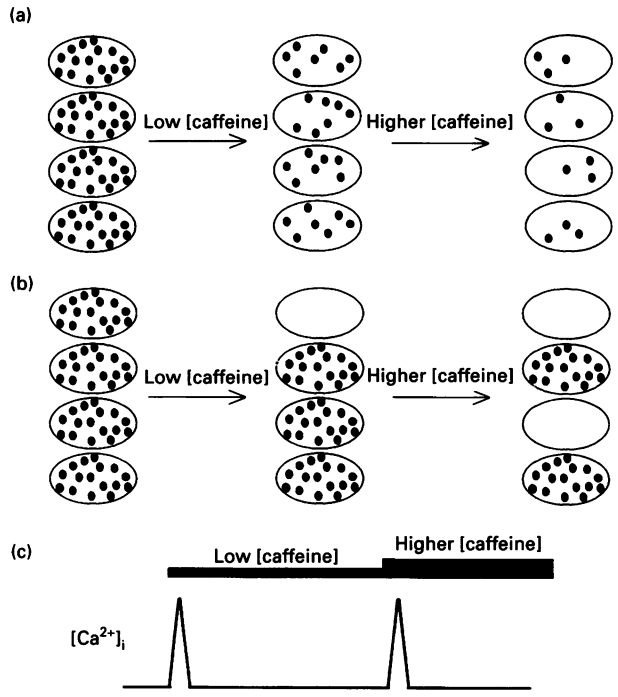

Figure 1 Quantal Ca^{2+} mobilization from RyR

Scheme showing how the quantal response (**c**) can be accounted for by (**a**) partial release from a functionally continuous store, or (**b**) all-or-none release from functionally discrete stores. The present data support an all-or-none release mechanism from functionally discrete stores (**b**), so for clarity the stores are also depicted as physically discrete. However, there is evidence suggesting that Ca^{2+} stores are able to remain functionally discrete even if physically continuous (Short et al., 1993).

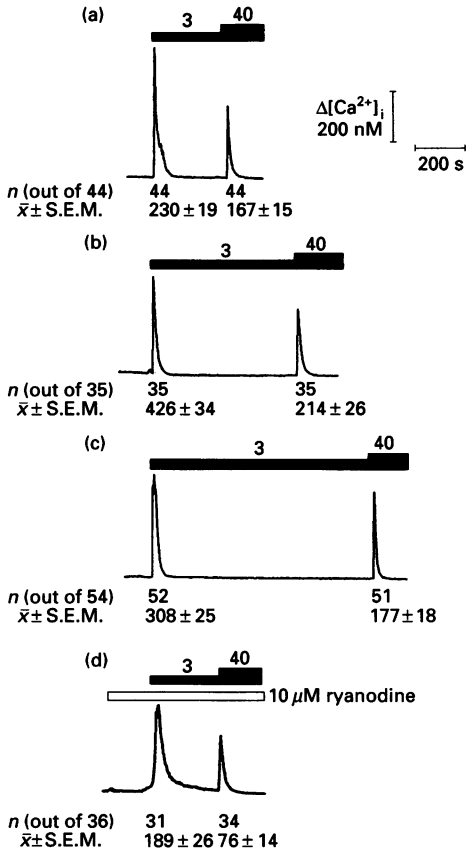

Figure 2 Quantal Ca^{2+} release from single chromaffin cells

(**a–c**) Fura-2-loaded chromaffin cells in nominally Ca^{2+}-free medium ($[Ca^{2+}]_o < 2\ \mu M$) were challenged with stepwise increases in caffeine concentration, as shown by the filled bars. Three independent cellular responses are presented, showing that the quantal Ca^{2+} release was independent of the interval between steps in caffeine concentration. The time between administration of 3 and 40 mM caffeine was (**a**) 5, (**b**) 10 and (**c**) 15 min. (**d**) Quantal Ca^{2+} release was also demonstrated in $[Ca^{2+}]_o$-containing medium, with ryanodine (10 μM) present. For each experiment, a representative response is shown, in addition to population data for the number of cells responding to each caffeine application (n) and the average $[Ca^{2+}]_i$ rise ($\bar{x}$) and the S.E.M.

response was observed when the 3 mM caffeine treatment was given in the presence of 10 μM ryanodine (Figure 2d). At this concentration, ryanodine is expected to bind to activated RyR and lock them into an open sub-conductance state (Stauderman and Murawsky, 1991; Buck et al., 1992; Cheek et al., 1993a). However, as the cells responded to both the initial 3 mM caffeine application and the subsequent addition of 40 mM caffeine, the data in Figure 2(d) reveal that the low caffeine concentration did not activate all the intracellular RyRs, since ryanodine had clearly not bound to the proportion of receptors that responded to 40 mM caffeine.

To investigate the mechanism underlying the quantal response we altered the experimental protocol from a sustained caffeine application to repetitive caffeine applications, as shown in Figure 3. The importance of using brief repetitive caffeine applications was that caffeine could be completely removed between applications, allowing $[Ca^{2+}]_i$ to return to the pre-stimulated level, so that any caffeine- or Ca^{2+}-dependent adaptation or inactivation of the RyR (Györke and Fill, 1993, 1994) could be reversed between caffeine applications. This is clearly the case since repetitive responses to caffeine were achieved (Figures 3a–3c). Consistent with this idea, Györke and Fill (1993) showed that the Ca^{2+}-dependent adaptation of cardiac RyR was reversed by reducing the Ca^{2+} concentration near the cytoplasmic surface of the RyR, allowing repetitive stimulations to be achieved every 30 s.

In the presence of an extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), each caffeine application resulted in an increase in $[Ca^{2+}]_i$ (Figures 3a–3c). However, in the absence of $[Ca^{2+}]_o$ (Figure 3d), or in the presence of 10 μM ryanodine (Figure 3e), the response to 40 mM caffeine progressively declined until the stores were depleted of Ca^{2+}. In the majority of cells, this depletion occurred within 1–3 caffeine pulses. As verification that the intracellular Ca^{2+} pool had been depleted under these conditions, pretreatment of chromaffin cell populations with 40 mM caffeine and 10 μM ryanodine inhibited the rise in $[Ca^{2+}]_i$ induced by the Ca^{2+}-ATPase inhibitor thapsigargin by ~ 96% (results not shown). These data suggest that in the presence of extracellular Ca^{2+}, the removal of caffeine allowed the caffeine-sensitive Ca^{2+} stores to refill with Ca^{2+} from the extracellular medium. They also suggest that Ca^{2+} released from the caffeine-sensitive stores was not significantly re-sequestered by the intracellular stores, since repetitive responses were not seen in the absence of $[Ca^{2+}]_o$. The ability of ryanodine to inhibit the repetitive responses within 1–3 caffeine applications revealed that it bound rapidly to activated RyR and prevented the stores from refilling.

In contrast to the total depletion of the caffeine-sensitive stores, achieved by 40 mM caffeine, repetitive applications of lower caffeine concentrations caused only partial depletion when

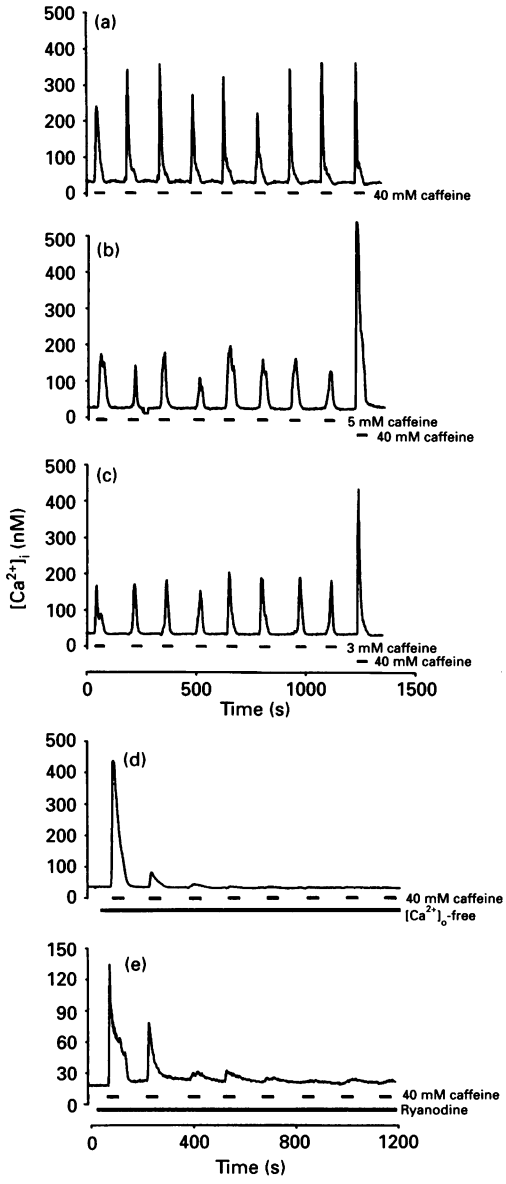

Figure 3 $[Ca^{2+}]_i$ responses in single chromaffin cells evoked by repetitive application of caffeine

Caffeine was applied for 30 s, with 180 s intervals. (**a–c**) Cells were repetitively stimulated with caffeine at the concentrations shown, in a $[Ca^{2+}]_o$-containing medium. (**d–e**) Repetitive stimulation with 40 mM caffeine in $[Ca^{2+}]_o$-free medium or in the presence of 10 μM ryanodine respectively.

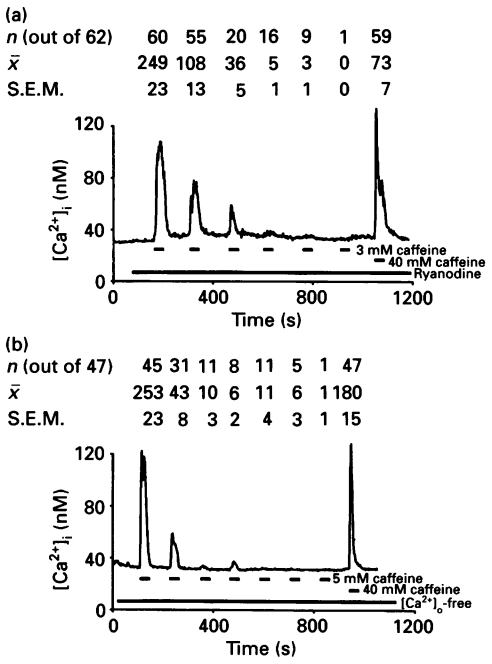

Figure 4 Low caffeine concentrations do not release the entire caffeine-sensitive Ca^{2+} pool

(**a**) 3 or (**b**) 5 mM caffeine was repetitively applied to chromaffin cells, either in $[Ca^{2+}]_o$-containing medium with 10 μM ryanodine (**a**), or $[Ca^{2+}]_o$-free medium (**b**). The population data are presented in a similar manner to Figure 2.

applied in the presence of 10 μM ryanodine (Figure 4a) or absence of $[Ca^{2+}]_o$ (Figure 4b). In both cases, after four applications of 3 or 5 mM caffeine respectively, the cells failed to respond, suggesting that the intracellular stores sensitive to these caffeine concentrations had been depleted. However, Ca^{2+} clearly remained in some intracellular stores, since the cells responded to the subsequent application of 40 mM caffeine.

This pattern of response is not predicted by a scheme in which the intracellular Ca^{2+} stores are functionally continuous. If the caffeine-sensitive stores were functionally continuous in chromaffin cells, i.e. the entire Ca^{2+} pool contains multiple homogeneously sensitive RyR (Figure 1a), repetitive applications of a low caffeine concentration in a Ca^{2+}-free medium would lead to a progressively diminishing response, eventually depleting the entire Ca^{2+} pool. Additionally, this model predicts that locking open only a few RyR by ryanodine would be expected to release all of the Ca^{2+} in the intracellular pool. However, our data show that when added with a low caffeine concentration, ryanodine locked open only those RyRs that were sensitive to the low dose of caffeine, since the cells responded when treated subsequently with a higher caffeine concentration (Figure 4a). Also, RyR adaptation was not responsible for the quantal release since the use of repetitive caffeine applications prevented a persistent caffeine- or Ca^{2+}-dependent RyR adaptation, as shown in Figures 3(a)–3(c).

These data, using repetitive caffeine applications, provide additional evidence that Ca^{2+} is released from caffeine-stimulated RyR in a quantal manner, as the higher caffeine concentration was able to mobilize more intracellular stores than the low dose. Additionally, they reveal that the intracellular caffeine-sensitive pool is composed of functionally discrete stores, bearing RyR that have heterogeneous sensitivities to caffeine. Individual stores can function independently of each other and when stimulated they release their Ca^{2+} in an all-or-none manner (Figure 1b).

All-or-none quantal Ca^{2+} release from intracellular stores that have heterogeneous sensitivities to their respective ligands may represent an important mechanism controlling cellular Ca^{2+} signals generated by CICR. Although a ubiquitous mechanism, CICR is paradoxical in that its inherent regenerative nature means that, once activated, it is expected to stimulate the complete release of the entire intracellular Ca^{2+} pool. *In vivo*, however, Ca^{2+} release from RyR is graded (Cannell et al., 1987; Cleemann and Morad, 1991; Sipido and Weir, 1991; Hua et al., 1993); subpopulations of intracellular stores behave independently (Oyamada et al., 1993) and release Ca^{2+} without necessarily

triggering a release from the remaining stores. A scheme in which Ca^{2+} is released in an all-or-none manner from stores that become progressively recruited as the stimulus intensity increases (Figure 1b) provides a mechanism by which regenerative release at the level of individual stores can be graded so as to give different amplitude responses at the level of the whole cell (cf. the effects of 3 versus 40 mM caffeine, Figure 3c). Such a mechanism could also explain how Ca^{2+} release in the heart can be graded by varying the amplitude of Ca^{2+} influx across the T-tubule membrane (Cleeman and Morad, 1991).

The demonstration of quantal Ca^{2+} release from RyR illustrates a functional similarity between IP_3R and RyR, although it is not yet clear whether the same mechanism, i.e. all-or-none release, applies to both types of receptor. A major tool in elucidating the mechanism of quantal release in this study was the use-dependent activity of ryanodine. Unfortunately, a similar agent does not yet exist for the IP_3R. However, the similarities between these receptors suggest that the conclusions drawn from this study on RyR could also apply to the mechanism underlying quantal release from IP_3R (Parker and Ivorra, 1990, 1993; Parker and Yao, 1991; Bootman et al., 1992). In order for a cell to display graded Ca^{2+} responses using an all-or-none quantal release mechanism from individual stores, the stores are required to be heterogeneously sensitive to their ligand. It is not clear how this heterogeneity is achieved, but possibilities include differences in the phosphorylation state of the intracellular receptors (Witcher et al., 1991; Burgess et al., 1991; Wang and Best, 1992), luminal Ca^{2+} content (Nelson and Nelson, 1990; Missiaen et al., 1991, 1992; Cheek et al., 1993a) or allosteric interactions between subunits in the tetrameric receptor complex (Lai et al., 1988; Chadwick et al., 1990). Whatever the cause, all-or-none quantal Ca^{2+} release from functionally discrete intracellular stores may be a mechanism that enables the generation of graded $[Ca^{2+}]_i$ signals at the level of the whole cell.

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