Calmodulin Dissociation Mediates Desensitization of the cADPR-Induced Ca^{2+} Release Mechanism

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Summary

Ryanodine receptor (RyR) activation by cyclic ADP-ribose (cADPR) is followed by homologous desensitization [1–3]. Though poorly understood, this "switching off" process has provided a key experimental tool for determining the pathway through which cADPR mediates Ca^{2+} release [4]. Moreover, desensitization is likely to play an important role in shaping the complexities of Ca^{2+} signaling involving cADPR, for example, localized release events [5, 6] and propagated waves [7–9]. Using the sea urchin egg, we unmask a role of calmodulin, a component of the RyR complex [10, 11] and a key cofactor for cADPR activity [12–14], during RyR/cADPR desensitization. Recovery from desensitization in calmodulin-depleted purified endoplasmic reticulum (microsomes) is severely impaired compared to that in crude egg homogenates. An active, soluble factor, identified as calmodulin, is required to restore the capacity of microsomes to recover from desensitization. Calmodulin mediates recovery in a manner that tightly parallels its time course of association with the RyR. Conversely, direct measurement of calmodulin binding to microsomes reveals a loss of specific binding during cADPR, but not IP$_3$, desensitization. Our results support a mechanism in which cycles of calmodulin dissociation and reassociation to an endoplasmic reticulum protein, most likely the RyR itself, mediate RyR/cADPR desensitization and resensitization, respectively.

Results and Discussion

As first described in sea urchin egg homogenates [1] and subsequently in mammalian systems [2, 3], the Ca^{2+} release mechanism activated by cADPR undergoes homologous desensitization. Since cADPR displays cross-desensitization with other activators of the RyR [2–4], desensitization can be envisaged to be occurring at either the level of store depletion or, alternatively, at the level of the RyR release machinery. The former may be less likely since both cADPR and IP$_3$ access overlapping endoplasmic reticulum stores [15] yet reveal no cross-desensitization. In the latter case, one possible mechanism might be the loss of a factor required for cADPR-mediated Ca^{2+} release from the RyR release complex.

Calmodulin is a candidate factor known to directly modulate RyR activity [11, 16–18] and sensitize cADPR-mediated Ca^{2+} release via direct interaction with the Ca^{2+} release machinery [12–14, 19].

To learn about the possible role of calmodulin during desensitization, we compared the properties of desensitization in sea urchin egg homogenate to those in microsomes, a Percoll-purified endoplasmic reticulum fraction depleted in calmodulin [12, 13]. Such experiments were possible since microsomes retained partial sensitivity to cADPR. This contrasts with previous reports in a different sea urchin species (*Strongylocentrotus purpuratus*) in which cADPR sensitivity was completely lost [12, 13], suggesting differences in the efficiency of removing endogenous factors (such as calmodulin) that confer sensitivity to cADPR-mediated release. Maximal Ca^{2+} release in both homogenates and microsomes was elicited by the addition of cADPR (500 nM). Subsequently, free Ca^{2+} in these closed systems returned to a basal level, reflecting Ca^{2+}-ATP-ase pump-mediated reuptake into Ca^{2+} stores [1]. At this point, both homogenate and microsome systems had entered a desensitized phase and were refractory to further additions of cADPR (Figure 1A).

Does this simply reflect emptying of the cADPR-sensitive pool via selective reuptake into other pools? *Lytichinus pictus* does not contain sufficient esterase to load esterified Ca^{2+} dyes, and so direct analysis of stored Ca^{2+} was not possible [20]. Instead, we exploited Ca^{2+} release elicited by IP$_3$ or the endoplasmic reticulum pump inhibitor thapsigargin as a tool to probe store loading. This was possible since, in the sea urchin egg, cADPR and IP$_3$ access Ca^{2+} from a common thapsigargin-sensitive endoplasmic reticulum store [15]. Further confirmation of a shared cADPR/IP$_3$ pool in our egg preparations arose from the observation that Ca^{2+} release by maximal cADPR and maximal IP$_3$ was nonadditive (data not shown). We find that maximum Ca^{2+} release by IP$_3$ and thapsigargin is unaltered in cADPR-desensitized microsomes, thus suggesting that the cADPR/IP$_3$ Ca^{2+} store is replete (i.e., Ca^{2+} is resequestered back into the pool from which it was released) at a time when the cADPR release mechanism is desensitized. Control Ca^{2+} release by IP$_3$ and thapsigargin in homogenates (500 μl) and microsomes (500 μl) was 10.1 ± 0.7 nmol Ca^{2+} (n = 3) and 8.8 ± 0.5 nmol Ca^{2+} (n = 3), respectively. Following resequestration of Ca^{2+} to basal levels after the addition of cADPR (500 nM), Ca^{2+} release by IP$_3$ in cADPR refractory homogenates and microsomes represented 85% ± 4.6% (n = 4) and 102% ± 3.1% (n = 4), respectively, of control. Similarly, total Ca^{2+} release by thapsigargin was only slightly reduced in cADPR-desensitized homogenates (91% ± 2.8% of control, n = 4) and was unaltered in microsomes (99% ± 3.0% of control, n = 4). That the cADPR release mechanism remains desensitized despite replenishment of its Ca^{2+} store is consistent with the locus of cADPR desensitization being independent of store loading.

We report that cADPR desensitization is transient.

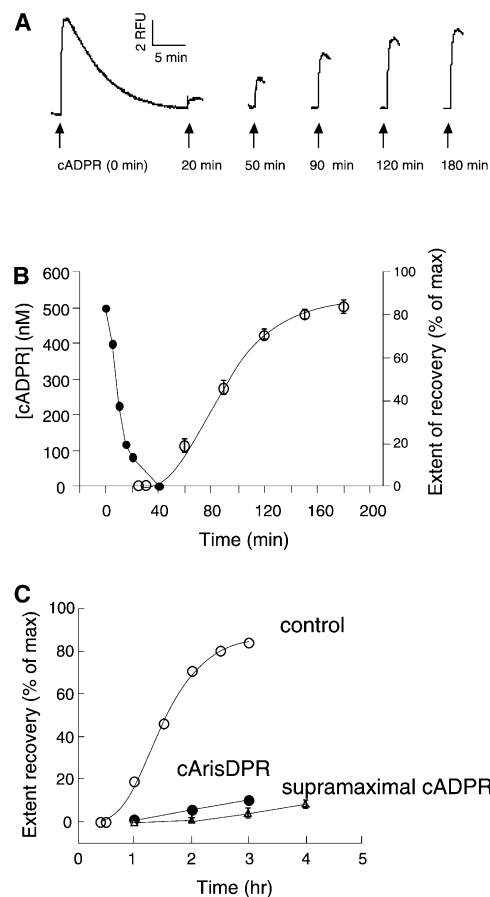[1]Correspondence: justyn.thomas@pharmacology.ox.ac.uk

Figure 1. Spontaneous Recovery of Crude Egg Homogenates from cADPR Desensitization Occurs following cADPR Degradation

(A) Representative fluorimetric traces illustrating Ca²⁺ release elicited by an initial desensitizing addition of cADPR (500 nM) and the subsequent time-dependent resensitization of homogenates rechallenged with cADPR (500 nM).

(B) A graph showing the time course of cADPR degradation (closed circles) and recovery of cADPR sensitivity (open circles) subsequent to the addition of cADPR (500 nM).

(C) A graph showing that resensitization to cADPR is severely reduced after homogenates are desensitized with either a hydrolysis-resistant cADPR analog, cArisDPR (500 nM) (closed circles), or a supramaximal (50 μM) concentration of cADPR (open triangles). For all experiments, the extent of recovery is expressed as the percentage of Ca²⁺ release elicited by the initial (desensitizing) addition of cADPR. Error bars represent mean ± standard error of 3–7 determinations.
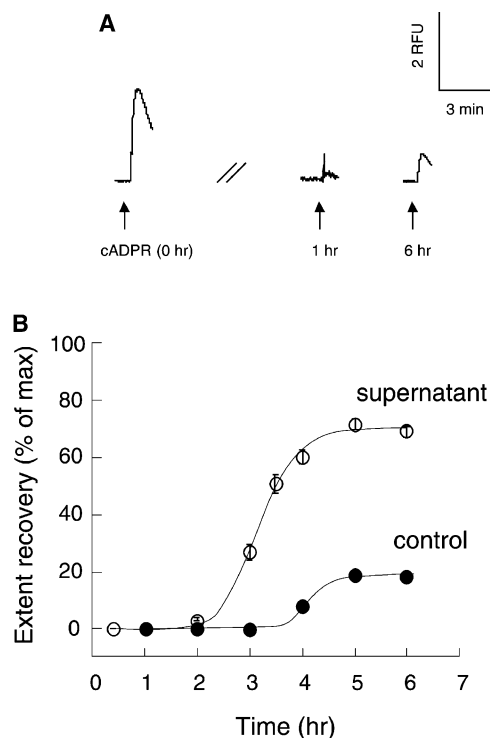
Figure 2. Recovery from Desensitization in Calmodulin-Depleted Microsomes Requires an Active Soluble Factor

(A) Representative fluorimetric traces illustrating Ca²⁺ release elicited by an initial desensitizing addition of cADPR (500 nM) and subsequent incomplete resensitization of microsomes rechallenged with cADPR (500 nM).

(B) A graph comparing the recovery from desensitization in control microsomes (open circles) and microsomes reconstituted with a Percoll-purified soluble fraction (filled circles). For each time point, the extent of recovery is expressed as the percentage of Ca²⁺ release elicited by the initial (desensitizing) addition of cADPR. Error bars represent mean ± standard error of 3–7 determinations.

Indeed, homogenates displayed time-dependent, spontaneous recovery to cADPR-induced Ca²⁺ release (Figure 1A). Comparison of the time course of recovery from desensitization to that of cADPR degradation indicates that initiation of recovery coincides with the near completion of cADPR hydrolysis by cADPR hydrolases to ADPR (Figure 1B). Thus, cADPR degradation appears to be prerequisite for spontaneous recovery. This contention was further supported by use of cyclic aristromycin diphosphate ribose (cArisADPR), a hydrolysis-resistant (half-time in sea urchin egg of ~150 min) cADPR analog of cADPR with similar Ca²⁺ releasing efficacy [21]. Recovery of sensitivity in homogenates

following desensitization with cArisADPR was minimal compared to recovery after desensitization with cADPR (Figure 1C). Similarly, use of a supramaximal concentration of cADPR (50 μM) also perturbed recovery (Figure 1C).

In contrast to crude homogenates, spontaneous recovery in purified microsomes was severely impaired. Maximal recovery represented ~20% (Figures 2A and 2B). This does not reflect the inability of microsomes to degrade cADPR, since cADPR hydrolase activity is greatest in this Percoll fraction and parallel functional studies revealed complete degradation of 500 nM cADPR by 2 hr (n = 5). Rather, this observation in a purified membrane preparation provides the first indication that a soluble factor, partially lost in this system, is required for the recovery process. It is of interest to note that dependence on a soluble factor would argue against operation of desensitization at the level of store depletion but rather suggests that this is intrinsic to the cADPR-activated release mechanism. Since cADPR cross-desensitizes with other activators of the RyR [4], this most likely concerns the RyR itself.

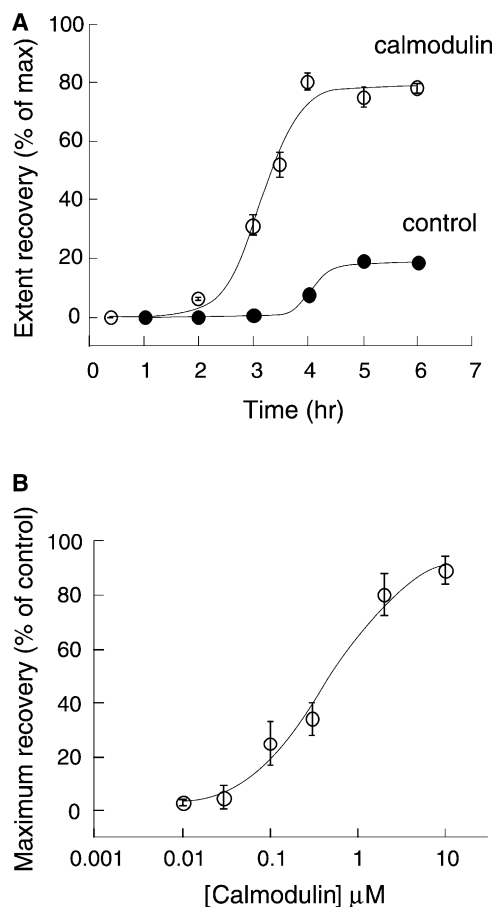Next, we directly tested the influence of calmodulin

Figure 3. Calmodulin Restores the Capacity of Microsomes to Recover from Desensitization

(A) The time course of recovery in aliquots of microsomes incubated with 2 μM calmodulin (open circles) or IM (filled circles) for 90 min (17°C) prior to the first (desensitizing) addition of cADPR (500 nM). (B) Concentration dependence of calmodulin-mediated resensitization of cADPR-activated Ca^{2+} release. The data are expressed as the percentage of Ca^{2+} release elicited by the initial (desensitizing) addition of cADPR. For all experiments, error bars represent mean $\pm$ standard error of 3–7 determinations.

Figure 4. Calmodulin Binding Decreases during cADPR Desensitization

(A) A histogram showing loss of calmodulin during cADPR, but not IP_3, desensitization in a manner inhibited by 8-amino cADPR. Following incubation with 1 μM [^{35}S]calmodulin for 3 hr (17°C), microsomes were treated with cADPR (500 nM) or IP_3 (2 μM) for 30 min. Parallel functional studies showed microsomes to be refractory to both messengers at this time point. Values are expressed as mean $\pm$ standard error of 3–4 determinations. An asterisk indicates the statistical significance against control (100%), as determined by ANOVA with subsequent separation means with Fisher's Least Significant Difference test, $p < 0.001$.
(B) The time course of [^{35}S]calmodulin binding during cADPR desensitization and resensitization. For all experiments, data are expressed as values relative to control in the presence of only [^{35}S]calmodulin, and nonspecific binding was determined with 10 μM unlabeled calmodulin. Values are expressed as mean $\pm$ standard error of 3–4 determinations.

on desensitization. This concerned reconstitution of microsomes with endogenous sea urchin egg calmodulin, enriched in the Percoll-purified supernatant fraction [12, 13], and exogenous calmodulin purified from bovine brain. Prior to these experiments, the time taken for calmodulin to mediate full potentiation of cADPR was elucidated. This was necessary since recovery was assessed by a comparison of cADPR-mediated Ca^{2+} release following desensitization to that elicited by the initial desensitizing cADPR. Such an approach enables possible modulation of recovery by calmodulin to be resolved from its overall potentiation of cADPR-mediated Ca^{2+} release. However, failure to allow sufficient time for calmodulin to mediate full potentiation (reflecting its association kinetics with the RyR) would cause an overestimation of subsequent recovery. Half-maximal and maximal potentiation of cADPR-mediated release in microsomes (incubated at 17°C) occurred at ~40 min and 80 min, respectively, after the addition of
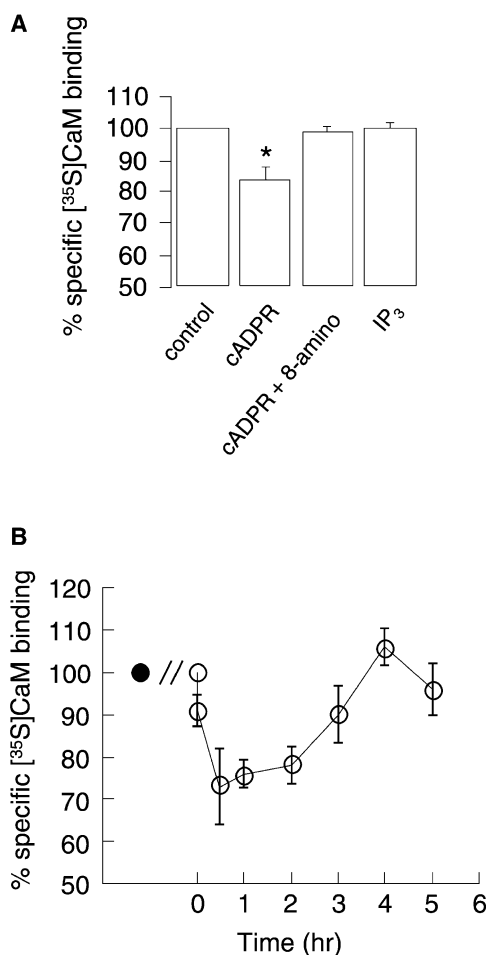
2 μM calmodulin. Thus, for all subsequent experiments, microsomes were incubated with calmodulin for 90 min prior to the first addition of desensitizing cADPR.

In agreement with previous reports [12, 13], the addition of calmodulin-enriched supernatant to purified microsomes enhanced initial release by maximal cADPR (500 nM). In addition, however, a dramatic near-complete recovery from desensitization was also observed (Figure 2B). The ability of supernatant to potentiate spontaneous recovery from desensitization was retained after heat treatment (100°C for 5 min). This is

consistent with the involvement of a heat stable factor, such as calmodulin. Nevertheless, use of supernatant alone cannot entirely exclude the possibility that another soluble factor is instead mediating recovery from desensitization. Importantly, however, the addition of purified, exogenous bovine brain calmodulin to microsomes precisely reproduced the effect of supernatant, restoring the capacity of microsomes to recovery from desensitization, with a half-maximal concentration of ~0.5 μM (Figures 3A and 3B).

Together, our results provide confirmation that calmodulin is the active factor that resensitizes the cADPR-activated release mechanism. The time course of calmodulin-mediated recovery in microsomes, following initial cADPR degradation, closely matches that of spontaneous recovery in crude homogenates. Moreover, this parallels the "apparent" association kinetics of calmodulin to the sea urchin RyR, which occurs with a half-maximal time of approximately 40 min, a value similar to that determined for calmodulin/RyR association in skeletal muscle (30 min) [11]. This is consistent with calmodulin mediating recovery of cADPR activity by association with the RyR release complex. However, since direct interaction of calmodulin with the RyR release machinery is required for the initial desensitizing cADPR-mediated release [12–14], this most likely occurs by reassociation with the RyR. In turn, this provides strong evidence that calmodulin dissociates from the RyR release complex during desensitization, possibly initiating this process.

Such a model predicts that loss of calmodulin binding would occur in response to a desensitizing cADPR treatment, and, therefore, it can be tested by using [^{35}S]calmodulin bound to Percoll-purified microsomes. As shown in Figure 4A, cADPR (500 nM) elicited a significant reduction in specific binding of [^{35}S]calmodulin in a manner that was abolished by its competitive antagonist, 8-amino cADPR (5 μM). To ensure that reduction in calmodulin binding was specific to cADPR, the effect of IP$_3$ on specific binding was also assessed. In contrast to cADPR, a maximal, desensitizing concentration of IP$_3$ (2 μM) had no effect on [^{35}S]calmodulin binding (Figure 4A). This not only argues against a similar role for calmodulin during IP$_3$ receptor desensitization but also eliminates the possibility that nonspecific effects of, for example, store depletion or elevation in free Ca^{2+}, are mediating the loss of calmodulin bindng. Specificity of reduction in calmodulin binding was further confirmed by an analysis of the time course of this process during cADPR desensitization. This revealed a pattern of loss and rebinding of [^{35}S]calmodulin to microsomes that precisely mirrored that of desensitization and resensitization, respectively (Figure 4B).

In summary, our results are consistent with cycles of calmodulin dissociation and reassociation to an endoplasmic reticulum-associated protein, most likely the RyR itself, mediating cADPR desensitization and resensitization, respectively. Thus, in addition to functioning as a cofactor for the initiation of cADPR-gated Ca^{2+} release, calmodulin appears to play a key role in "switching off" this process. This not only confirms an intimate role for calmodulin in shaping the complexities of cADPR signaling but also marks a novel mechanism for inactivation of intracellular Ca^{2+} signaling.

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