Pharmacological characterization of the putative cADP-ribose receptor

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cADP-ribose (cADPR), a naturally occurring metabolite of NAD⁺, has been shown to be an important regulator of intracellular Ca²⁺ release. Considerable evidence suggests that cADPR is the endogenous modulator of the ryanodine receptor (RyR), which mediates Ca²⁺-induced Ca²⁺ release (CICR). Indeed, cADPR-mediated Ca²⁺ release is subject to functional regulation by other modulators of CICR, including Ca²⁺, caffeine and calmodulin. However, the underlying basis behind the effect of such agents on cADPR activity (in particular whether they regulate cADPR binding), as well as the precise nature of the cADPR receptor remains unclear. In the present study, use of ³²P-radiolabelled cADPR has enabled a detailed pharmacological characterization of cADPR-binding sites in sea urchin egg homogenates. We report that cADPR binds specifically to a single class of high affinity receptor. Retainment of binding to membranes after a high-salt wash suggests the involvement of

either an integral membrane protein (possibly the RyR itself) or a peripheral protein tightly associated to the membrane. Insensitivity of [³²P]cADPR binding to either FK506 or rapamycin suggests that this does not concern the FK506-binding protein. Significantly, binding is highly robust, being relatively insensitive to both endogenous and pharmacological modulators of RyR-mediated CICR. In turn, this suggests that such agents modulate cADPR-mediated Ca²⁺ release primarily by tuning the 'gain' of the CICR system, upon which cADPR acts, rather than influencing the interaction of cADPR with its target receptor. The exception to this is calmodulin, for which our results indicate an additional role in facilitating cADPR binding.

Key words: Ca²⁺-induced Ca²⁺ release, calmodulin, ryanodine receptor.

INTRODUCTION

Changes in cytosolic Ca²⁺ provide an important signalling mechanism for the regulation of a great diversity of cellular functions, and release of Ca²⁺ from intracellular stores plays a major role in generating these signals [1]. cADP-ribose (cADPR), a naturally occurring metabolite of NAD⁺, has been shown to be an important regulator of intracellular Ca²⁺ release. Indeed, subsequent to the first report of its activity as the unidentified Ca²⁺-releasing NAD⁺ metabolite in sea urchin eggs [2], the Ca²⁺-mobilizing activity of cADPR, together with the enzymic machinery for its synthesis and degradation, has been found in a wide variety of tissues from plants through to many mammalian systems [3,4].

The pharmacology of cADPR-induced Ca²⁺ release is consistent with a mechanism that involves sensitization of the ryanodine receptor (RyR) to the process of Ca²⁺-induced Ca²⁺ release (CICR). Activators of CICR, including caffeine and divalent cations, potentiate cADPR- but not inositol 1,4,5-trisphosphate (IP₃)-induced Ca²⁺ release from sea urchin microsomes [5,6]. Moreover, caffeine displays cross-desensitization with cADPR with respect to Ca²⁺ release [7] and conversely, pharmacological blockers of CICR, such as procaine and Ruthenium Red, specifically inhibit cADPR-sensitive Ca²⁺ release [8]. Further evidence for activation of the RyR has been the demonstration that cADPR-mediated Ca²⁺ release is greatly enhanced by calmodulin [9], a protein associated with the RyR complex [10] and which can act to sensitize CICR [11–13]. However, the underlying basis behind the effect of CICR modulators on cADPR activity, in particular whether they regulate cADPR binding, is unclear.

It is also currently unknown whether cADPR mediates Ca²⁺ release by direct association with the RyR in a manner analogous

to IP₃ with its receptor, or indirectly, by binding a RyR-associated accessory protein. cADPR has previously been shown to specifically bind saturable sites on purified microsomes from sea urchin (*Strongylocentrotus purpuratus*) eggs [14]. Use of photo-affinity labelling with cADPR analogues, however, has indicated two specific binding proteins with molecular masses significantly smaller than that of known RyRs [15]. The size of these proteins is much lower than any known RyRs and may represent degradation products for the RyR or alternatively RyR-associated accessory proteins. Consistent with the latter, a candidate receptor for cADPR is the FK506-binding protein. Indeed, a study in pancreatic islets [16] has suggested that cADPR may activate the RyR by relieving the FK506-binding-protein-12.6-mediated inhibition.

To gain further insights into the nature of the cADPR receptor, the aim of the present study was to pharmacologically characterize the binding of [³²P]cADPR to sea urchin (*Lytechinus pictus*) egg homogenate. We demonstrate that [³²P]cADPR binds specifically to a single class of high affinity receptor, in a manner relatively insensitive to both endogenous and pharmacological modulators of RyR-mediated CICR. Thus, the primary mechanism by which such agents modulate cADPR-mediated Ca²⁺ release appears to operate by tuning the sensitivity of the RyR to CICR, upon which cADPR then acts, rather than influencing the interaction of cADPR with its target receptor. The exception to this is calmodulin, for which our results indicate an additional role in facilitating cADPR binding.

MATERIALS AND METHODS

Materials

β-[³²P]NAD⁺ was from Amersham. All other chemicals were from Sigma. *L. pictus* sea urchins were obtained from Marinus

Abbreviations used: cADPR, cADP-ribose; CICR, Ca²⁺-induced Ca²⁺ release; IM, intracellular medium; IP₃, inositol 1,4,5-trisphosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; RyR, ryanodine receptor.

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(Long Beach, CA, U.S.A.). Eggs were obtained by stimulating the ovulation of female sea urchins with an intracoelomic injection of KCl. Egg jelly was removed by filtration through a nylon mesh (pore diameter 90 μ m), and eggs were then washed twice in artificial sea water (435 mM NaCl, 15 mM MgSO₄, 11 mM CaCl₂, 10 mM KCl, 2.5 mM NaHCO₃ and 1.0 mM EDTA, pH 8.0).

Preparation of sea urchin eggs and microsomes

Homogenates of sea urchin eggs were prepared as described previously [17]. In addition to crude homogenate, microsomes were purified from 50% (v/v) egg homogenates by Percoll density centrifugation. Briefly, a fractionating buffer was prepared by diluting Percoll stock to 25% in a modified intracellular medium (IM) (333 mM *N*-methyl glucamine, 333 mM potassium acetate, 27 mM Hepes and 1.3 mM MgCl₂; pH titrated to 7.2 with acetic acid), supplemented with 0.5 mM ATP, 2 units/ml creatine phosphokinase, 4 mM phosphocreatine and 10 μ M EGTA. Then, 1.25 ml of 50% egg homogenate was layered on 9 ml of this solution and centrifuged at 27000 *g* for 30 min at 15 °C in thin-walled plastic tubes. The Ca²⁺-storing microsomes form a distinct tight band approximately half-way down the tube, and 1 ml of this was collected using a disposable syringe and needle to puncture the vessel wall. The fractions were stored at -80 °C until use.

Synthesis of [³²P]cADPR

The tracer [³²P]cADPR was synthesized from a high specific activity (1000 Ci/mmol) radiolabelled form of its precursor β -[³²P]NAD⁺. The cyclization reaction was carried out for 2 h at room temperature (approx. 22 °C) using 250 μ Ci of β -[³²P]NAD⁺, 100 ng/ml ADP-ribosyl cyclase and 5 mM Tris/HCl, pH 7.4, in a total volume of 250 μ l. cADPR was purified using an HPLC method as described previously [14]. Fractions containing cADPR were neutralized with Tris base and then stored at -20 °C.

[³²P]cADPR binding

Sea urchin egg homogenate was made up to a final concentration of 0.5 mg/ml protein in IM buffer (250 mM *N*-methyl glucamine, 250 mM potassium gluconate, 20 mM Hepes and 1 mM MgCl₂, pH 7.2). Approx. 22.5 fmol of [³²P]cADPR, together with various concentrations of unlabelled test compound, were incubated with homogenate at a total volume of 250 μ l for 10 min (unless otherwise stated) at room temperature (approx. 22 °C). The binding reaction was terminated by rapid filtration with a Brandel cell harvester using GF/B filters washed immediately before filtration with ice-cold IM, and washed twice with 2–4 ml of ice-cold IM immediately after filtration. Radioactivity retained on the filters was determined using standard scintillation counting techniques. Results were corrected for non-specific binding, as assessed by the addition of 10 μ M unlabelled cADPR.

Data analysis

Curves were fitted using the Hill equation (by the least-squares method):

$$S = S_0/[1 + [C]/IC_{50}]^h$$

where IC₅₀ is the concentration of competitor (*C*) causing half-maximal inhibition of specific [³²P]cADPR binding (*S*), *S*₀ is specific binding in the absence of competitor and *h* is the Hill

coefficient. All results are expressed as the means $\pm$ S.E.M. of *n* values.

RESULTS

Specificity of binding

Time-dependent association of enzymically synthesized [³²P]cADPR to sea urchin egg homogenate is shown in Figure 1(A). As shown in Figure 1(B), binding occurred in a manner inhibited by increasing concentrations of unlabelled cADPR, consistent with the presence of a saturable receptor for cADPR. The plot of competitive displacement in Figure 1(B) is consistent with a single class of binding site with an affinity (*K*_d) of 2.1 ± 0.14 nM, a capacity (*B*_{max}) of 12 fmol/mg of protein and a Hill coefficient of 0.97 ± 0.05 . As previously shown in *S. purpuratus* [14], the specificity of binding was illustrated by the fact that NAD⁺, the precursor of cADPR, and ADPR, the hydrolysis product, were less effective in competing for the [³²P]cADPR-binding site (Figure 1C). In the present study, the effect of ATP, AMP, IP₃ and nicotinic acid adenine dinucleotide phosphate (NAADP) was also investigated. None of these agents produced displacement of [³²P]cADPR at similar concentrations to cADPR (Figure 1C).

We next examined the effects of cADPR analogues on [³²P]cADPR binding. As shown in Figure 2, specific binding of [³²P]cADPR was eliminated by incubation with the specific cADPR antagonists 8-amino-cADPR and 8-bromo-cADPR, as well as cADPR-phosphate, a cyclized metabolite of NADP with Ca²⁺-mobilizing activity in mammals [18], but not sea urchin eggs (results not shown). Importantly, the rank order of potency (8-amino-cADPR > 8-bromo-cADPR > cADPR-phosphate) shows both 8-amino-cADPR and 8-bromo-cADPR to be dramatically more effective than cADPR-phosphate, with regard to inhibition of [³²P]cADPR (Figure 2).

Regulation of binding by pH and KCl

Figure 3(A) shows that radiolabelled-ligand binding to egg homogenate occurred maximally at approx. pH 6.5, but was inhibited under both acidic and alkaline conditions. As reported previously [14], this is in contrast to the cerebellar IP₃ receptor, which shows an increase in binding with increasing pH until a plateau is reached at pH 9–9.5 [19]. Figure 3(B) shows that [³²P]cADPR binding to Percoll-purified microsomes was inhibited by KCl in a concentration-dependent manner. The inhibitory effect of high salt (1.2 M KCl) was completely reversible after washing the membranes in control IM, whereby specific binding was 98.3 ± 4.0 (*n* = 3) of control. Reversible inhibition was also observed with NaCl treatment (results not shown).

Effect of divalent cations on [³²P]cADPR binding

The major regulator of the RyR/Ca²⁺-release channel is Ca²⁺ itself. As shown in Figure 4(A), [³²P]cADPR binding was relatively insensitive to [Ca²⁺], whereby high micromolar concentrations of Ca²⁺ only weakly inhibited total [³²P]cADPR binding. A closer examination of binding in the range between 100 nM to 1 μ M Ca²⁺ was also performed. Precise adjustment of Ca²⁺ concentration was performed using the pH-metric method as described previously [20]. Importantly, the affinity of binding remained unaltered across this range (Figure 4B). High micromolar concentrations of Mg²⁺ and Sr²⁺ were also found to weakly inhibit [³²P]cADPR binding as shown in Figure 4(C).

Figure 1 Specific [³²P]cADPR binding to sea urchin (*L. pictus*) egg homogenates

(A) Association kinetics of [³²P]cADPR binding. *L. pictus* homogenates were incubated at 21 °C with 22.5 fmol of [³²P]cADPR for the indicated times prior to the determination of the bound radiolabelled ligand. (B) Binding of [³²P]cADPR to homogenates in the presence of the indicated concentrations of unlabelled cADPR. Homogenates were incubated with unlabelled and radiolabelled cADPR for 10 min prior to determination of bound radiolabelled ligand. (C) Specificity of [³²P]cADPR binding. Specific binding of [³²P]cADPR to egg homogenate was determined in the presence of various concentrations of cADPR (●), ADPR (○), NAD (▲), NAADP (△), ATP (◇), ADP (◆), IP₃ (×). Homogenates were incubated with specified unlabelled ligand and [³²P]cADPR for 10 min prior to determination of bound radiolabelled ligand. Results are expressed as values relative to control in the presence of only [³²P]cADPR. For all experiments non-specific binding was determined using 10 μM unlabelled cADPR. Results are expressed as the means ± S.E.M. for 4–6 experiments.

Figure 2 Competitive-equilibrium cADPR binding

Competitive displacement of [³²P]cADPR by the cADPR analogues: 8-amino-cADPR (●), 8-bromo-cADPR (○) and cADPR-phosphate (◆). The results are expressed as values relative to control in the presence of [³²P]cADPR only. For all experiments non-specific binding was determined using 10 μM unlabelled cADPR. Results are expressed as the means ± S.E.M. for 6–9 experiments.

Figure 3 Sensitivity of [³²P]cADPR binding to pH and KCl

(A) Sensitivity of [³²P]cADPR binding to both high and low pH. The pH of homogenates was adjusted by adding small aliquots of either acetic acid or KOH. The results are expressed as values relative to control in the presence of [³²P]cADPR only. (B) Inhibition of [³²P]cADPR binding by KCl. Homogenates were exposed to the NaCl and [³²P]cADPR simultaneously and incubated for 10 min prior to determination of bound radiolabelled ligand. Results are expressed relative to specific binding in the absence of KCl. For all experiments non-specific binding was determined using 10 μM unlabelled cADPR. Results are expressed as the means ± S.E.M. for 6–9 experiments.

Figure 4 Effect of divalent cations on [³²P]cADPR binding

(A) Specific binding of [³²P]cADPR to egg homogenates was determined in the presence of various concentrations of Ca²⁺. The results are expressed as values relative to control in the presence of only [³²P]cADPR. (B) The affinity of [³²P]cADPR binding remains unchanged between 100 nM (◆), 300 nM (○) and 1 μM (●) Ca²⁺. (C) Effect of Sr²⁺ (●) and Mg²⁺ (○) on [³²P]cADPR binding. The results are expressed as values relative to control in the presence of [³²P]cADPR only. For all experiments non-specific binding was determined using 10 μM unlabelled cADPR. Results are expressed as the means ± S.E.M. for 6–9 experiments.

Rapamycin and pharmacological modulators of the RyR do not effect [³²P]cADPR binding

Since a candidate receptor for cADPR is the RyR-associated FK506-binding protein [16], the effect of the FK506-binding protein inhibitor, rapamycin on [³²P]cADPR binding was examined. Rapamycin is a highly potent inhibitor of the FK506-binding protein, mediating full inhibition at the sub-nanomolar range [21]. In the present study, concentrations of rapamycin up to 30 μM failed to alter [³²P]cADPR binding (Figure 5A). Similarly, in the presence of 30 μM FK506, specific binding was

96.9 ± 1.1% (*n* = 3) of control, suggesting that at least in the urchin egg the FK506-binding protein is not the receptor mediating cADPR-induced Ca²⁺ release.

The effect of caffeine and ryanodine on competitive displacement of [³²P]cADPR binding by unlabelled cADPR is shown in Figures 5(B) and 5(C) respectively. Neither of these agents altered the affinity or strongly inhibited [³²P]cADPR binding. In the presence of 10 mM, 2 mM and 1 mM caffeine, specific cADPR binding represented 69.5 ± 3.3% (*n* = 6), 93.8 ± 7.0% (*n* = 6) and 96.2 ± 4.7% (*n* = 6) of the control respectively. Specific [³²P]cADPR binding in the presence of 600 μM ryanodine represented 92.0 ± 2.5% of control. Two representative CICR inhibitors are Ruthenium Red and tetracaine [22]. Neither of these agents significantly reduced [³²P]cADPR binding over concentration ranges that inhibit cADPR/RyR-mediated Ca²⁺ release, even at their respective maximal inhibitory concentrations (Figure 5D). Indeed, the concentration of each agent required to reduce binding was far in excess of that required to inhibit cADPR-mediated Ca²⁺ release, and so interference with cADPR binding would not appear to underlie their inhibitory effects on cADPR-induced Ca²⁺ release.

Modulation of [³²P]cADPR binding by calmodulin

Calmodulin is an endogenous modulator of CICR- and cADPR-mediated Ca²⁺ release in the sea urchin egg. The influence of calmodulin on specific [³²P]cADPR binding was examined using the antagonists, calmidazolium, W7 and suramin [23]. Calmidazolium inhibited specific [³²P]cADPR binding in a concentration-dependent manner (IC₅₀ = 68.7 ± 14.4 μM, *n* = 6) whereby maximal inhibition (1 mM calmidazolium) reduced specific binding to 22.9 ± 2.4% (*n* = 6) of control (Figure 6A). Similarly, W7 (1 mM) and suramin (30 μM) reduced specific binding to 32.9 ± 6.5% (*n* = 6) and 11.0 ± 3.0% (*n* = 4) of control respectively (results not shown). It has been reported previously [9] that cADPR sensitivity of Percoll-purified microsomes, in which calmodulin is depleted, can be fully restored by re-addition of calmodulin. A direct examination of whether calmodulin alters the affinity of cADPR binding was performed by assessing the effect of calmodulin on the competitive displacement of [³²P]cADPR by unlabelled cADPR in Percoll purified microsomes. Microsomes were incubated at room temperature (approx. 22 °C) with 1 μM calmodulin for 1 h, prior to addition of unlabelled and radiolabelled cADPR. It is important to note that the effect of calmodulin on binding affinity varied between Percoll-purified microsomes prepared from different batches of homogenates. Figure 6(B) shows results collated from two separate batches, in which incubation of microsomes with 1 μM calmodulin significantly increased the binding affinity of [³²P]cADPR. Microsomes from a third batch of homogenate, however, did not display a significant calmodulin-induced shift in binding affinity. A possible explanation for the differential sensitivity to calmodulin between preparations may be varying degrees of calmodulin depletion during the isolation process. Indeed, the basal affinity of [³²P]cADPR binding to the 'calmodulin-insensitive' microsomes was higher than the 'calmodulin-sensitive' microsomes (*K_d* = 4.1 ± 1.7 and 12.1 ± 3.0 nM respectively), suggesting that comparatively higher levels of endogenous calmodulin remained bound.

Effect of store loading on [³²P]cADPR binding

In addition to cytosolic regulators of Ca²⁺ release, the level of Ca²⁺ within the lumen of the endoplasmic reticulum also adjusts the activity of RyRs [24]. [³²P]cADPR binding was assessed in

Figure 5 Insensitivity of [³²P]cADPR binding to pharmacological modulators of CICR

(A) Specific binding of [³²P]cADPR to egg homogenates was determined in the presence of various concentrations of rapamycin. The results are expressed as values relative to control in the presence of only [³²P]cADPR. Effect of the RyR agonists (○), (B) caffeine (2 mM) and (C) ryanodine (80 μM), on control (●) competitive displacement of binding by unlabelled cADPR. (D) Effect of Ruthenium Red (●) and tetracaine (○) on [³²P]cADPR binding. Results are expressed as values relative to control in the presence of only [³²P]cADPR. For all experiments non-specific binding was determined using 10 μM unlabelled cADPR. Results are expressed means ± S.E.M. for 6–9 experiments.

homogenates loaded with Ca²⁺ (in the presence of an ATP-regenerating system) and homogenates treated with the selective Ca²⁺ ATPase inhibitor thapsigargin (10 μM). The properties of [³²P]cADPR binding to both Ca²⁺-loaded (*n* = 3) and thapsigargin-treated homogenates (*n* = 3) were identical to untreated homogenates (results not shown).

DISCUSSION

The use of enzymically synthesized ³²P-labelled cADPR has enabled the detailed characterization of a single class of high affinity cADPR receptor in sea urchin (*L. pictus*) eggs. Importantly, the low nanomolar affinity of binding to this site is in the physiological range of cADPR-induced Ca²⁺ release, consistent with the identification of the site through which this messenger mediates Ca²⁺ mobilization in the sea urchin egg. Further confirmation that [³²P]cADPR binds to physiologically relevant receptors arises from use of cADPR analogues, the effect of which on binding strongly correlates with their functional effects on cADPR activity. Indeed, the rank order of potency of a series of cADPR analogues shows two potent antagonists of cADPR-mediated release, 8-amino-cADPR and 8-bromo-cADPR, to be dramatically more effective than cADPR-phosphate with regard to the competition of [³²P]cADPR binding. Alongside cADPR, two other messengers, IP₃ and NAADP, also release Ca²⁺ in sea

urchin eggs. In agreement with Lee [14], IP₃ was ineffective in competing for radiolabelled ligand binding in the sea urchin egg homogenate. Another messenger, NAADP [25], also failed to markedly reduce [³²P]cADPR binding. These results provide further evidence that the release mechanism activated by cADPR is independent of other Ca²⁺-mobilizing messengers reported to operate in the sea urchin egg. Yet, the precise nature of the cADPR-binding protein remains uncertain. Retainment of binding to membranes after high-salt wash suggests the involvement of either an integral membrane protein (such as the RyR) or a peripheral protein tightly associated with the membrane. A well characterized RyR accessory protein and a candidate receptor for cADPR is the FK506-binding protein [16]. However, the binding to egg homogenates was insensitive to rapamycin, a potent inhibitor of this protein, and FK506, suggesting that at least in the urchin egg the FK506-binding protein is not the receptor mediating cADPR-induced Ca²⁺ release.

Since cADPR acts by increasing the sensitivity of the Ca²⁺-dependent activation mechanism of the RyR-channel protein, a defining feature of cADPR activity is its sensitivity to other agents that influence this process including Ca²⁺ itself, other divalent cations, caffeine, calmodulin and RyR antagonists, such as Ruthenium Red. However, the underlying basis behind the effect of such agents on cADPR-induced Ca²⁺ release is unclear. Indeed, it has yet to be established whether they simply tune the

Figure 6 Regulation of [32 P]cADPR binding by calmodulin

(A) Concentration-dependent inhibition of [32 P]cADPR binding by the calmodulin antagonist calmidazolium. Results are expressed as values relative to control in the absence of calmidazolium. Results are expressed as mean $\pm$ S.E.M. for 4–6 experiments. (B) Competitive displacement of [32 P]cADPR binding by unlabelled cADPR in Percoll-purified microsomes, in the presence (●) and absence (○) of calmodulin (1 μ M). Microsomes were incubated at approx. 22 °C with calmodulin for 1 h prior to addition of unlabelled and radiolabelled cADPR. For all experiments non-specific binding was determined using 10 μ M unlabelled cADPR. Results are expressed as the means $\pm$ S.E.M. for 4–9 experiments.

sensitivity of the RyR machinery to CICR, upon which cADPR then acts, or additionally influence the interaction of cADPR with its target proteins. The principal endogenous regulator of RyR channel is Ca^{2+} . Activating, as well as inhibitory, Ca^{2+} -binding sites are found within the sequence of such channel proteins [26]. Investigation of the effect of this ion on the interaction of cADPR with its receptor revealed only modest inhibition at high micromolar concentrations. Such inhibition, however, appeared to be a general effect of high divalent cation concentrations, rather than a specific effect of Ca^{2+} . Thus at resting (0.2 μ M) and RyR-activating (1–10 μ M) Ca^{2+} concentrations Ca^{2+} would not appear to influence the interaction of cADPR with its receptor. This is in contrast to divalent ion potentiation of cADPR-mediated Ca^{2+} release [5,11]. Only in the vicinity of an open channel would the Ca^{2+} reach a sufficient level (100–300 μ M) to weakly reduce cADPR binding, possibly providing a negative feedback on cADPR-mediated release. Therefore these results suggest that regulation of cADPR binding is not a major factor underlying Ca^{2+} control of cADPR-mediated Ca^{2+} release. From analysis of the potentiating effects of caffeine on cADPR-mediated release in the sea urchin egg homogenate, it has been shown that caffeine alters the apparent affinity of cADPR to its receptor [5,27]. Since the molecular binding sites of cADPR and caffeine are expected to differ (8-amino-cADPR blocks cADPR- but not caffeine-induced Ca^{2+} release) the basis of any such

regulation would be expected to occur allosterically. However, direct assessment of the effect of caffeine on [32 P]cADPR binding in the present study has not revealed regulation of cADPR binding by caffeine. Thus, the shift in apparent affinity of cADPR reported in functional studies more likely reflects caffeine-mediated sensitization of CICR, upon which cADPR then acts, rather than an increase in affinity of cADPR to its target protein. Similarly the inhibitory effect of the CICR inhibitors, such as Ruthenium Red and tetracaine, on cADPR-mediated Ca^{2+} release would not appear to operate by interference with cADPR binding, since the concentration of these agents required to mediate inhibition of [32 P]cADPR binding is far in excess of that required to mediate functional inhibition of cADPR-mediated Ca^{2+} release.

In contrast to other modulators of RyR-mediated CICR, the present study has identified the possible involvement of calmodulin in modulating cADPR binding in the sea urchin egg. Calmodulin acts as an endogenous cofactor for cADPR-mediated Ca^{2+} release in the sea urchin egg [7], and has been shown to act synergistically with cADPR to sensitize RyR-mediated CICR [11]. Examination of the influence of calmodulin on cADPR binding in the present study suggests that in addition to sensitization of CICR, calmodulin promotes cADPR binding to its receptor. Indeed, [32 P]cADPR binding to sea urchin egg homogenate displays a marked sensitivity to calmodulin antagonists, including calmidazolium. This result correlates with the requirement of calmodulin for cADPR-activated Ca^{2+} release [9], and suggests that at least one effect of this protein is facilitation of either direct cADPR binding or assembly of a cADPR-binding accessory protein with the RyR release channel complex. Since our results indicate that addition of calmodulin to Percoll-purified microsomes enhances the affinity of [32 P]cADPR binding, this is consistent with promotion of cADPR association with existing receptors rather than recruitment of additional binding sites. Such activity is likely to be occurring through non-enzymic means, since the cADPR-sensitizing effects of calmodulin has been shown to occur via direct interaction with the Ca^{2+} -release mechanism itself [9].

In summary, these observations suggest that binding of cADPR to the sea urchin egg homogenate is remarkably robust. Thus functional modulation of cADPR by RyR/CICR modulators does not appear to operate by influencing the interaction of cADPR with its receptor. Instead, it is likely that by tuning RyR sensitivity to CICR, possibly by exposing or occluding either activating or inhibitory Ca^{2+} -binding sites, such agents tune the responsiveness of the RyR to cADPR, an endogenous modulator of CICR. The exception to this is calmodulin, which together with sensitizing the process of CICR may also play an additional role in facilitating cADPR binding. This in turn might imply the close proximity of calmodulin to the cADPR-binding site, whether this is found on the RyR itself or a tightly associated accessory protein.

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