7-Deaza cyclic adenosine 5′-diphosphate ribose: first example of a Ca^{2+}-mobilizing partial agonist related to cyclic adenosine 5′-diphosphate ribose

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Background: Cyclic adenosine 5′-diphosphate ribose (cADPR), a naturally occurring metabolite of nicotinamide adenine dinucleotide (NAD^+), mobilizes Ca^{2+} from non-mitochondrial stores in a variety of mammalian and invertebrate tissues. It has been shown that cADPR activates ryanodine-sensitive Ca^{2+}-release channels, working independently of inositol 1,4,5-trisphosphate (IP_3) to mobilize intracellular Ca^{2+} stores. In some systems, cADPR has been shown to be more potent than IP_3. The chemo-enzymatic synthesis of structurally modified analogues of cADPR can provide pharmacological tools for probing this new Ca^{2+}-signaling pathway. In this work, we describe the synthesis and evaluation of a structural mimic of cADPR with different Ca^{2+}-releasing properties.

Results: 7-Deaza cyclic adenosine 5′-diphosphate ribose (7-deaza cADPR), a novel cADPR analogue modified in the purine ring, was synthesized and its ability to release Ca^{2+} from non-mitochondrial pools in homogenates made from sea urchin eggs was investigated. 7-Deaza cADPR was more effective in releasing Ca^{2+} than cADPR, but it only released approximately 66% of the Ca^{2+} released by a maximal concentration of cADPR. It was also more resistant to hydrolysis than cADPR. If we administered increasing concentrations of 7-deaza cADPR at the same time as a maximal concentration of cADPR, the induction of Ca^{2+} release by cADPR was antagonized.

Conclusions: 7-Deaza cADPR has a Ca^{2+}-release profile consistent with that of a partial agonist, and it is the first reported example of such a compound to act at the cADPR receptor. The imidazole ring of cADPR is clearly important in stimulating the Ca^{2+}-release machinery, and the present results demonstrate that structural modification of a site other than position 8 of the purine ring can affect the efficacy of Ca^{2+} release. 7-Deaza cADPR represents a significant step forwards in designing modulators of the cADPR signaling pathway.

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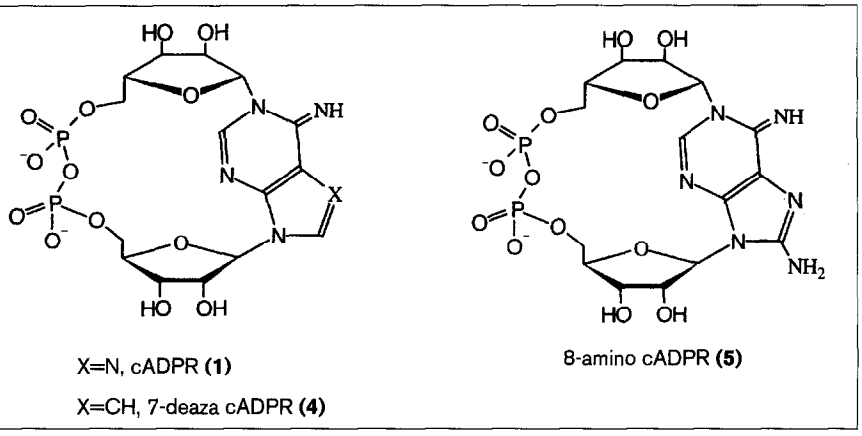
Introduction

Cyclic adenosine 5′-diphosphate ribose (cADPR, **1**, Fig. 1) is a candidate second messenger that mobilizes Ca^{2+} from non-mitochondrial stores in a variety of mammalian and invertebrate tissues [1–3]. The metabolic steps known to synthesize [4] and degrade [5] cADPR are understood and its N^1-ribosylated cyclic structure has been confirmed by X-ray crystallography [6]. The compound is of particular interest because it is known to be more effective than, and to work independently of, inositol 1,4,5-trisphosphate in mobilizing internal stores of Ca^{2+} in sea urchin eggs. It was in sea urchin eggs that its Ca^{2+}-releasing activity was first discovered [7]. Enzymes that synthesize and degrade cADPR are widely expressed in mammalian tissues, and in human lymphocytes, CD38 — a surface antigen — acts as a bifunctional enzyme for both synthesizing and degrading cADPR [8,9].

The exact mechanism by which cADPR mobilizes Ca^{2+} is still unknown. Initial evidence led to the proposal that, cADPR modulates the ryanodine receptor and because it sensitizes a ryanodine-sensitive Ca^{2+}-release mechanism to activation by divalent cations [10], it is an endogenous modulator of calcium-induced calcium release (CICR). In addition, it has recently been discovered that the binding of calmodulin to the ryanodine receptor can confer increased sensitivity on the cADPR-modulated CICR mechanism [11]. But cADPR may also serve as a potential second messenger, and such an action has been proposed to be involved in insulin release [3]. This idea is supported by the ability of nitric oxide (NO) and 3′,5′-cyclic guanosine monophosphate (cGMP) to mobilize intracellular Ca^{2+} release in sea urchin eggs by stimulating cADPR synthesis [12,13]. It has also been reported that the cholecystokinin octapeptide can augment the synthesis of

Figure 1



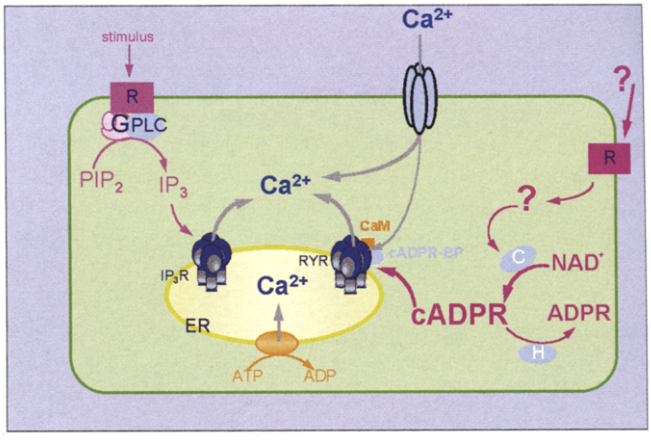
The structures of cADPR (**1**), 7-deaza cADPR (**4**) and 8-amino cADPR (**5**).

cADPR in mammalian intestinal longitudinal smooth muscle in a concentration-dependent manner [14]. A schematic representation of the Ca^{2+}-signaling pathways in sea urchin eggs is shown in Figure 2.

The importance of structurally modified analogues of cADPR as pharmacological tools for the further investigation of its biological properties is readily apparent. In order to produce such compounds most effectively we, like others [15], have relied upon a chemo-enzymatic approach to prepare cADPR itself and to generate structural diversity (see Fig. 3 for the synthesis of 7-deaza cADPR) [16]. A key requirement of this approach in the synthesis of structural analogues of cADPR is the loose substrate-specificity of the enzyme which cyclizes β-nicotinamide adenine dinucleotide (β-NAD^+). Nucleosides and their analogues are thus selectively phosphorylated and the resultant nucleotides (e.g. **2**, Fig. 3) are chemically coupled, yielding either β-NAD^+ or an appropriate analogue (e.g. **3**, Fig. 3). The product is cyclized enzymatically by *Aplysia* ADP-ribosyl cyclase in a third step to yield either natural cADPR (**1**, Fig. 1) or a modified cADPR analogue, respectively (e.g. **4** and **5**, Fig. 1). As well as being used for the synthesis of a number of sugar and purine modified analogues, this approach has also led to the identification of the first poorly hydrolyzed analogue of cADPR, cyclic aristeromycin 5′-diphosphate ribose (cArisDPR) [17].

Using synthetic modifications, we have identified a number of cADPR-like agonists [16]. Previously, 8-amino cADPR (**5**, Fig. 1) had been shown to behave as a competitive antagonist in inhibiting cADPR-induced Ca^{2+} release [15]. No partial antagonists or partial agonists have yet been identified, however. There is, therefore, considerable interest in studying further chemical modifications at position 8 of cADPR, in order to explain the structural requirements for this antagonism and to design a better antagonist. It also seems logical that this antagonism could

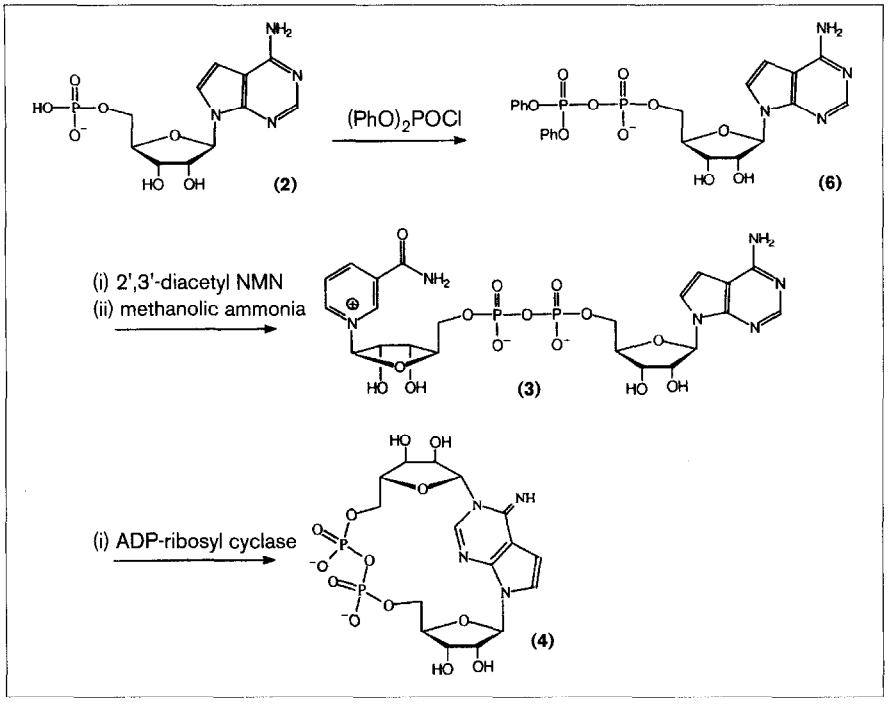
Figure 2



Schematic diagram to show the Ca^{2+}-release mechanisms in sea urchin eggs. In the IP_3 pathway, phosphatidylinositol 4,5-bisphosphate (PIP_2) is cleaved by G-protein-coupled phospholipase C (PLC), in response to stimulation of a receptor (R), to generate the Ca^{2+}-mobilizing second messenger IP_3; there is also direct influx of Ca^{2+} from the extracellular medium, and a putative cADPR-mediated second messenger pathway. The release of Ca^{2+} by cADPR is a complex process and is not yet well understood. A series of signals from an extracellular receptor may result in the cyclization of β-NAD^+ by ADP-ribosyl cyclase (C) to give cADPR. This acts in some way on the ryanodine receptor (RYR) on an internal Ca^{2+} store and results in Ca^{2+} release. The action requires calmodulin (CaM), however, and may involve the interaction of cADPR binding protein (cADPR-BP) with the Ca^{2+}-release channel. There are two distinct ways in which cADPR might act: first, as a second messenger analogous to IP_3, with extracellular stimuli ultimately initiating the cyclization process or, second, cADPR might act as a modulator for CICR. In the second model, the level of cADPR in the cytoplasm acts to sensitize the ryanodine receptor to increases in $[Ca^{2+}]_i$. Thus, both cADPR and a rise in the internal concentration of cellular Ca^{2+} are required to trigger CICR. The increase in intracellular Ca^{2+} may come from an influx through a plasma membrane channel or from release via the IP_3 mechanism, where IP_3 acts on its receptor (IP_3R) on the endoplasmic reticulum (ER). The breakdown of cADPR to ADPR is mediated by the enzyme cADPR-hydrolase (H).

Figure 3

The chemo-enzymatic route used to synthesize analogues of cADPR, showing the structures of 7-deazaadenosine 5′-monophosphate (**2**), activated 7-deazaadenosine 5′-monophosphate (**6**), 7-deaza NAD^+ (**3**) and 7-deaza-cADPR (**4**).



be studied further by exploring the effects of change at adjacent positions in the molecule. We have therefore investigated the effect of modification at position 7 of cADPR. As the first example of this unique modification, we have synthesized 7-deaza cADPR (**4**, Fig. 1) using a chemo-enzymatic route. 7-Deaza cADPR is a purine modified analogue of cADPR, in which the N7 atom of the adenine moiety has been replaced by a carbon atom. Preliminary modeling studies indicated that this compound had a very similar conformation to cADPR itself (data not shown). The pharmacological characteristics of this analogue were evaluated in sea urchin egg homogenates, in a comparative study with cADPR. We report here that whereas 7-deaza cADPR, like cADPR, can induce Ca^{2+} release from sea urchin egg homogenates, it is less efficacious and represents the first cADPR-based partial agonist. These results have implications for the design of new cADPR antagonists and for the mechanism of cADPR activation of its Ca^{2+}-release machinery.

Results

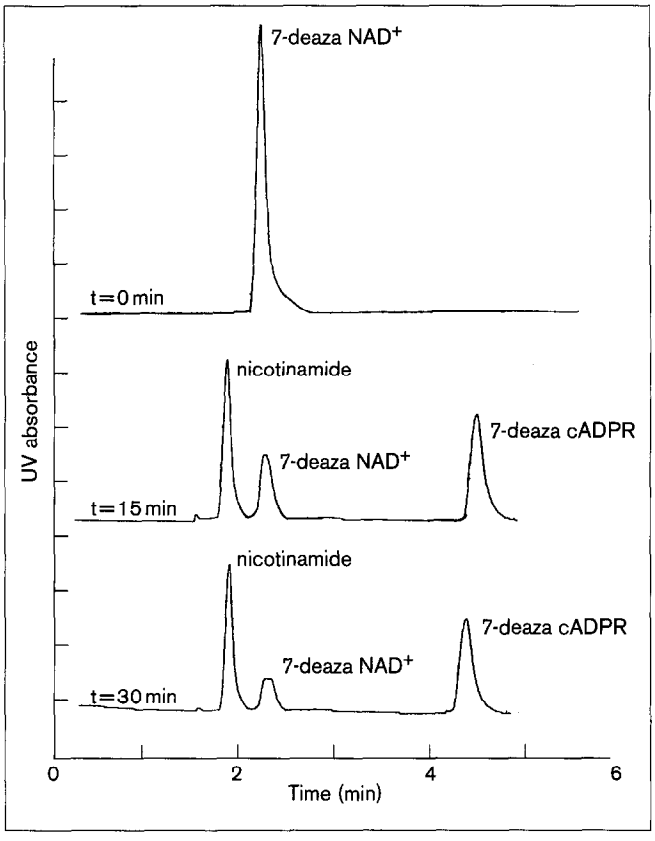
Chemo-enzymatic synthesis of 7-deaza cADPR

7-Deaza adenosine 5′-monophosphate (**2**, Fig. 3) was prepared using established methods to selectively phosphorylate the 5′-hydroxyl group. In order to minimize complications in the subsequent phosphate-coupling step, it was necessary to ensure that the product was free of contaminating inorganic phosphate. This was achieved by adsorption of the nucleotide on to activated charcoal, washing with water to remove the inorganic material, and elution of the product with aqueous ethanolic ammonia. ^{31}P NMR spectroscopy showed that the product was free from contaminant as there was only a single broad peak which sharpened on broad band proton decoupling (data not shown).

The coupling of adenosine 5′-monophosphate analogues to nicotinamide mononucleotide (NMN) using dicyclohexylcarbodiimide [18] and 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide-HCl [15] has previously been used to synthesize β-NAD^+ analogues. We favoured the more chemically flexible method of Michelson, however [19]. The 7-deaza cADPR described above was activated by forming a mixed anhydride intermediate (**6**, Fig. 3) using diphenyl chlorophosphate (DPPC) in dry dioxane-DMF. After evaporation, the residue was dissolved in a mixture of dry DMF and dry pyridine and treated with the phosphate monoanion of NMN. The protected 2′,3′-diacetyl derivative of NMN had to be used in order to overcome the lack of solubility of the unprotected NMN in the reaction solvent. Displacement of the acidic diphenyl phosphate moiety gave a pyrophosphate product which, after deprotection with methanolic ammonia and subsequent purification by ion-exchange chromatography, led to 7-deaza NAD^+ (**3**, Fig. 3).

ADP-ribosyl cyclase catalyzes the cyclization of β-NAD^+ to produce cADPR [4] and the *Aplysia* enzyme has been shown to be quite tolerant with respect to the purine base of its substrate [15,16]. Consequently, enzymatic

Figure 4



Monitoring the cyclization of 7-deaza NAD+ by HPLC. The enzymatic cyclization was carried out as described in the Materials and methods section: **(a)** t=0 min, 7-deaza NAD+ 100% at 2.19 min; **(b)** t=15 min, 7-deaza cADPR is seen at 4.87 min and the by-product nicotinamide is at 1.80 min; **(c)** t=30 min, the percentage of 7-deaza cADPR had increased only nominally so the reaction was quenched immediately.

cyclization of 7-deaza NAD+ was performed according to the protocol previously detailed from this laboratory [16]. The formation of the cyclized product, 7-deaza-cADPR (**4**, Fig. 1), was monitored by high performance liquid chromatography (HPLC; Fig. 4). The retention times for 7-deaza NAD+, 7-deaza cADPR and the by-product nicotinamide were 2.19 min, 4.87 min and 1.80 min, respectively, and the reaction was assumed to be complete when the peak assigned to nicotinamide was no longer increasing. The cyclization mixture was quenched by dilution after 30 min, a similar reaction time to that required for cADPR itself. 7-Deaza cADPR was easily purified from a small amount of unreacted starting material and nicotinamide by ion-exchange chromatography.

The UV spectrum of 7-deaza cADPR has a maximum at 272 nm (compared to 7-deaza adenosine 266 nm) whereas cADPR has a maximum at 259 nm [20]. The extinction coefficient for 7-deaza cADPR at 272 nm and at pH 7.0 was determined by total phosphate analysis of the cyclic nucleotide as $8150\,M^{-1}cm^{-1}$ compared to $12200\,M^{-1}cm^{-1}$ for 7-deaza adenosine. The value of the extinction coefficient for 7-deaza cADPR was used to determine the concentrations of 7-deaza cADPR used in the rest of the study. The proton NMR spectrum of the cyclized product showed the characteristic shift of the *2′* ribose proton of the adenosine moiety: $\delta = 5.4$ ppm for 7-deaza cADPR, a movement from the 4.4 ppm for 7-deaza NAD+. The shift was analogous to that described for cADPR itself [21]. Fast atom bombardment mass spectrometry, also confirmed cyclization: a mass of 539 for the negative molecular ion indicated the loss of nicotinamide.

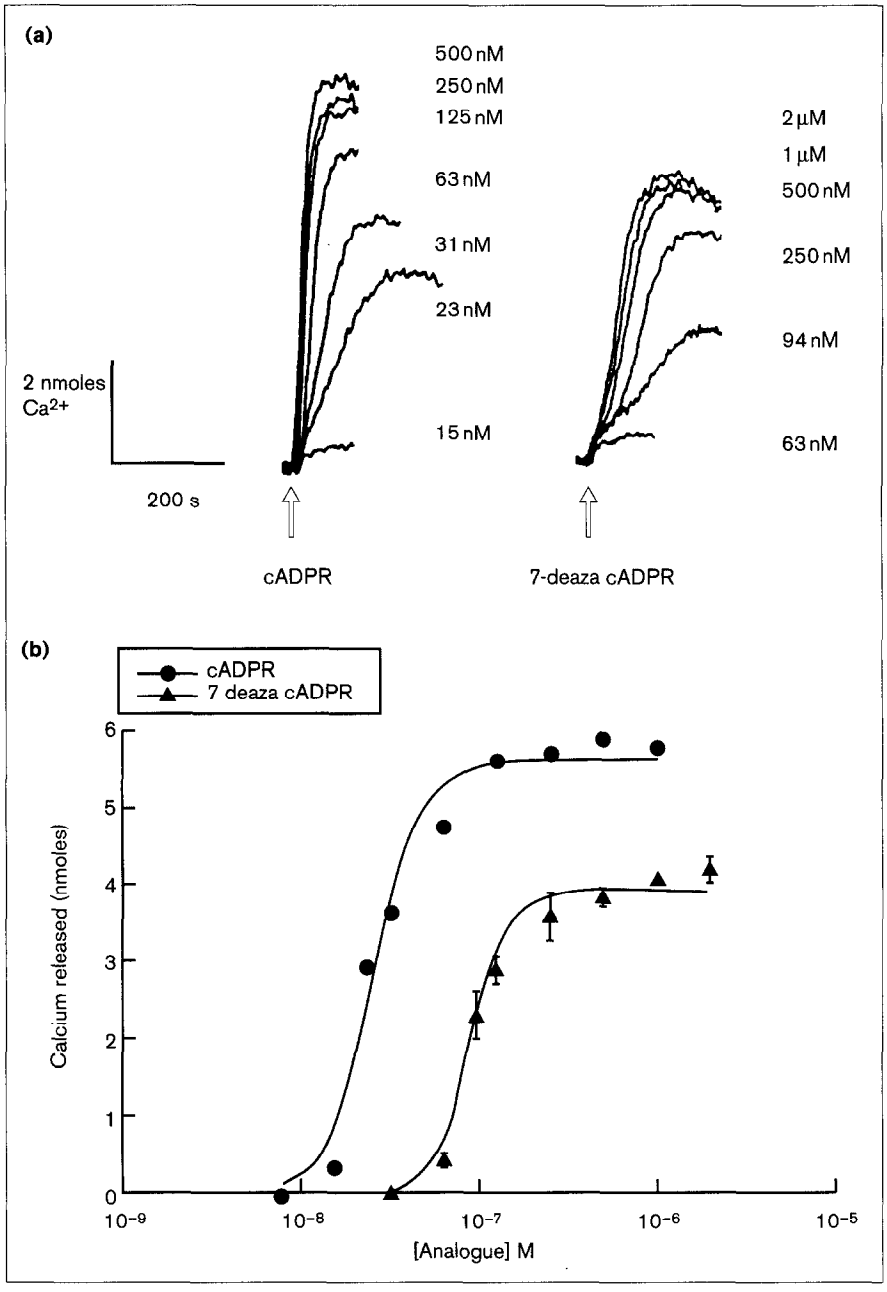
The Ca^{2+}-releasing ability of 7-deaza cADPR

The potency of concentration-dependent Ca^{2+} release by 7-deaza cADPR was assayed in sea urchin egg homogenate to determine whether this novel compound would be effective at releasing Ca^{2+} from such stores. As shown in Figure 5, 7-deaza cADPR was capable of releasing Ca^{2+} but the amount of Ca^{2+} released was consistently less than that released by the same concentration of the parent compound. The half-maximum Ca^{2+} release (EC_{50}) induced by cADPR and 7-deaza cADPR were 25 ± 2 nM, SEM $n = 3$ and 90 ± 4 nM, SEM $n = 3$, respectively. At maximal concentrations 7-deaza cADPR was able to release only around 66% of the Ca^{2+} mobilized by a maximal concentration of cADPR. Furthermore, this effect was unlikely to be due to an enhanced hydrolysis of the analogue by cADPR hydrolases because the half-time for degradation for the 7-deaza analogue was considerably longer than for cADPR. This indicated that the 7-deaza analogue was resistant to hydrolysis by cADPR hydrolases. (Fig. 6a and inset). 7-Deaza cADPR was also tested for its resistance to chemical hydrolysis. After heating a solution of cAPDR or 7-deaza cADPR to 85°C for 2 h, the samples were tested for their ability to release Ca^{2+}. 7-Deaza cADPR was shown not to have lost any releasing ability, whereas the sample of cADPR had been rendered inactive (Fig. 6b). HPLC analysis of these samples (conditions as described in the Materials and methods section) also confirmed that 7-deaza cADPR was still present in the sample but that all the cADPR had been destroyed. In a similar experiment, 7-deaza cADPR and cADPR were heated separately in HEPES buffer pH 7.3 (20 mM) at 85°C for 15 h. A comparative HPLC study showed that whereas all the cADPR had degraded, levels of 7-deaza ADPR had fallen by only 22% (data not shown).

The apparent lower intrinsic activity of 7-deaza cADPR indicated that it might be a partial agonist in the cADPR Ca^{2+}-release system. This was tested by examining the ability of 7-deaza cADPR to inhibit the maximal Ca^{2+} mobilizing properties of cADPR. The maximal Ca^{2+} released by cADPR alone was seen to be decreased by increasing the concentration of 7-deaza cADPR (Fig. 7a). Hence, 7-deaza cADPR and cADPR can progressively compete for Ca^{2+}-release activity, and cADPR can eventually overcome the

Figure 5

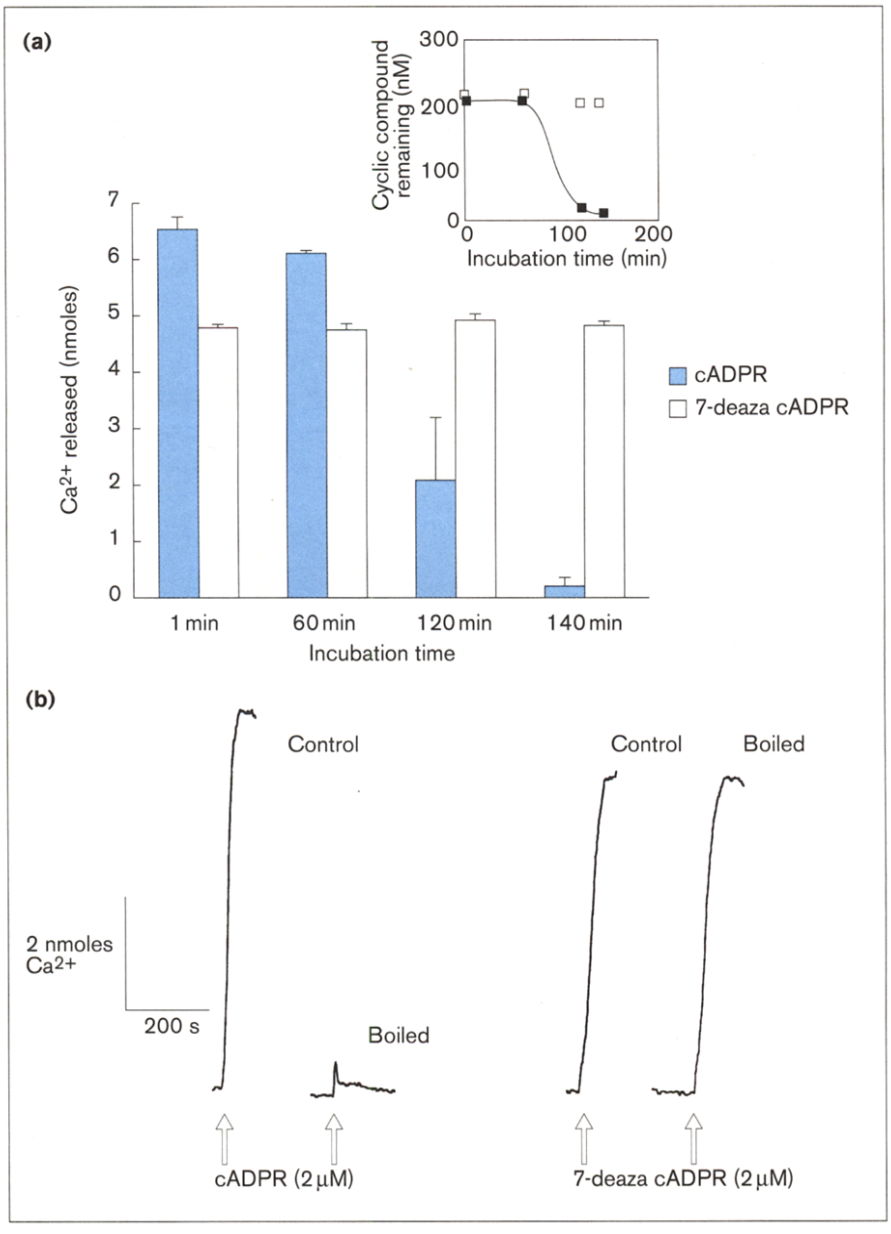
The Ca^{2+}-releasing action of cADPR and 7-deaza cADPR on sea urchin egg microsomes. *Lytechinus pictus* egg homogenate (2.5%) was prepared and challenged with increasing concentrations of cyclic compounds as described in the Materials and methods section.
(a) Representative Ca^{2+}-release profiles showing the concentration-dependent release of Ca^{2+} as detected by Fluo-3 fluorescence.
(b) Concentration–response curves of cADPR and 7-deaza cADPR (see key on figure). The amount of Ca^{2+} release was estimated as described previously [19]. The concentrations yielding half the maximal activity (EC_{50}) were estimated to 25 ± 2 nM for cADPR and 90 ± 4.4 nM for 7-deaza cADPR.



inhibition produced by a maximally effective concentration of 7-deaza cADPR. Combinations of submaximal concentrations of cADPR and 7-deaza-cADPR appeared to be additive, however, (Fig. 7b) so it is unlikely that an inhibitory metabolite is formed to affect cADPR-induced Ca^{2+} release. These data are consistent with a partial agonist action of 7-deaza cADPR.

Cross desensitization experiments verified that 7-deaza cADPR acts at the same Ca^{2+}-release channel as cADPR. Homogenate that was pre-treated with a supra-maximal dose of cADPR (1 μM) showed no further Ca^{2+} release when subsequently treated with 7-deaza cADPR (500 nM), and *vice versa* (Fig. 8). In addition, when homogenate was treated with 8-amino cADPR (250 nM), a competitive antagonist of cADPR-induced Ca^{2+} release [15], no further Ca^{2+} release was elicited by subsequent addition of either cADPR (500 nM) or 7-deaza cADPR (500 nM). Binding studies of [^{3}H]-cADPR to sea urchin egg homogenates further confirmed that 7-deaza cADPR and cADPR interact with the same binding site. 7-Deaza cADPR was equally as effective as cADPR at reducing radio-ligand binding at comparable concentrations, indicating similar affinities for the same binding site (Fig. 9).

Figure 6



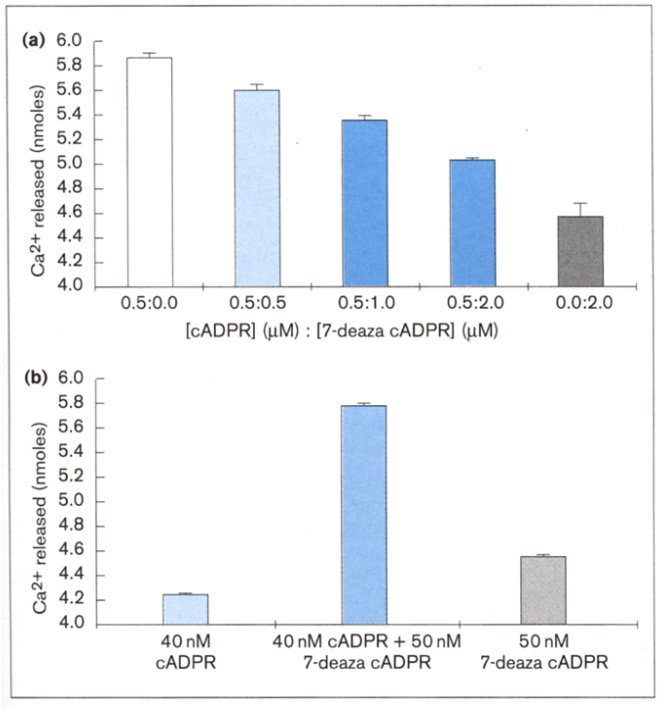
Stability of 7-deaza cADPR. **(a)** A comparison of hydrolysis of cADPR and 7-deaza cADPR in sea urchin egg homogenates. Egg homogenates were prepared for fluorimetry as described in the Materials and methods section. Egg homogenates (2.5%) were incubated at 17°C with 2 μM cADPR or 7-deaza cADPR. At the times indicated, 50 μl samples were taken and immediately assayed for the presence of a Ca^{2+}-mobilizing agonist (in 500 μl untreated homogenate). The starting concentration in the assay cuvette was therefore 200 nM for each agonist. The amount of Ca^{2+} released was estimated as previously described [20]. Values represent means of three experiments ± standard error (SE). The inset shows the same data, with the Ca^{2+} release recalibrated from the concentration–response curves in Figure 5b, showing predicted changes in concentrations of 7-deaza cADPR and cADPR as a function of incubation time. **(b)** Fluorimetric traces showing the action of heat-induced hydrolysis on Ca^{2+}-mobilizing activities of cADPR and 7-deaza cADPR. Both compounds (200 μM) were heated in a water-bath to 85–90°C for 2 h; 5 μl samples were taken before and after heat treatment and assayed for Ca^{2+}-releasing ability in 2.5% egg homogenate. Traces are representative of three determinations. Whereas the Ca^{2+}-mobilizing activity of cADPR (2 μM in the assay cuvette) was completely abolished following heat treatment, the activity of 7-deaza cADPR (similarly 2 μM in the assay cuvette) remained unaffected.

Discussion

Recently, there has been intense interest in the involvement of cADPR in the mechanism of Ca^{2+} release within cells. The realization that cADPR-induced Ca^{2+} mobilization operates in a wide number of different tissue types and cell lines [22], including T cells [23,24] and cardiac myocytes [25], has added considerable impetus to this field. Clearly, in order to improve our understanding of this system and elucidate the nature of the receptor for cADPR, structure–activity studies are urgently required. Until now, however, little work using structurally modified analogues of cADPR, other than modifications at position 8, has been reported.

The demonstration of the loose substrate specificity of *Aplysia* cyclase, the cyclizing enzyme, has reduced the problem of synthesizing cADPR analogues broadly to one of synthesizing the corresponding β-NAD^+ analogues — a much more straightforward chemical task. In fact, it is reasonable to suggest that the only major limiting factor involves modification to the pyrimidine ring. For example, the use of nicotinamide guanine dinucleotide and nicotinamide hypoxanthine dinucleotide leads to N7 ribosylation, not N1 ribosylation (our unpublished observations and see [26]). Conversely, all modifications to the imidazole ring and the adenosine ribose that we have made have been accepted by the enzyme.

Figure 7



Action of 7-deaza cADPR as a partial agonist. Egg homogenates were prepared for fluorimetry as described in the Materials and methods section. **(a)** The maximal amount of Ca^{2+} released from sea urchin egg microsomes by 500 nM cADPR was decreased when it was added at the same time as increasing concentrations of 7-deaza cADPR. **(b)** The effect of co-addition of submaximal concentrations of the two agonists. The values represent mean of triplicates ±SE.

The overall chemical strategy is, therefore, to prepare, by synthesis or otherwise, a structurally modified adenosine nucleoside, and then to phosphorylate this compound directly and regioselectively using P(V) chemistry. Subsequent coupling of the product to NMN leads to the corresponding β-NAD^+ analogue and enzymatic cyclization of this analogue leads in turn to an analogue of cADPR. The recent advent of the poorly hydrolyzable analogue of cADPR, cArisDPR [17], using this chemo-enzymatic strategy is a powerful demonstration of the immediate utility of the method. Use of this analogue in place of cADPR may highlight the presence of cADPR in tissues, which has not yet been detected, perhaps because of its rapid hydrolysis.

A starting point for the systematic study of the structure–activity relationships and the design of new antagonists is clearly the modification of position 8 of cADPR, because 8-amino cADPR is already documented as a competitive antagonist [15,20]. As groups at position 8 have such a dramatic influence on Ca^{2+}-mobilizing activity the adjacent position 7 is also an obvious target for chemical modification. We therefore decided to delete the N7 atom of the imidazole ring. Modeling of this compound showed it to be of a similar conformation to cADPR. When a comparison of the pharmacology of 7-deaza cADPR and cADPR was made, however, our results demonstrated that 7-deaza cADPR had a Ca^{2+}-release profile consistent with it being a partial agonist. The release of Ca^{2+} by 7-deaza cADPR indicated that the N7 atom is not essential for receptor binding and activation. Nevertheless, the absence of the N7 atom clearly interfered with the Ca^{2+}-release machinery, such that a submaximal release was observed. It is difficult at present to rationalize this observation in molecular terms, but the observation that cADPR is a full agonist and 7-deaza cADPR is a partial agonist may potentially reflect a requirement for hydrogen bonding at position N7 of the

Figure 8

7-Deaza cADPR acts on cADPR-sensitive Ca^{2+}-release channels. Egg homogenates were prepared for fluorimetry as described in the Materials and methods section. Representative Ca^{2+}-release profiles for both **(a)** 7-deaza cADPR and **(b)** cADPR show that both compounds release Ca^{2+} via the same channels in sea urchin egg homogenate (2.5%). Pretreatment of the homogenate with the cADPR antagonist 8-amino cADPR (250 nM) completely inhibited subsequent release by 500 nM 7-deaza cADPR and substantially decreased that induced by 500 nM cADPR. Receptors desensitized by pretreatment with a supra-maximal concentration of cADPR inhibited further Ca^{2+} release by a subsequent addition of 500 nM 7-deaza cADPR, and *vice versa*.

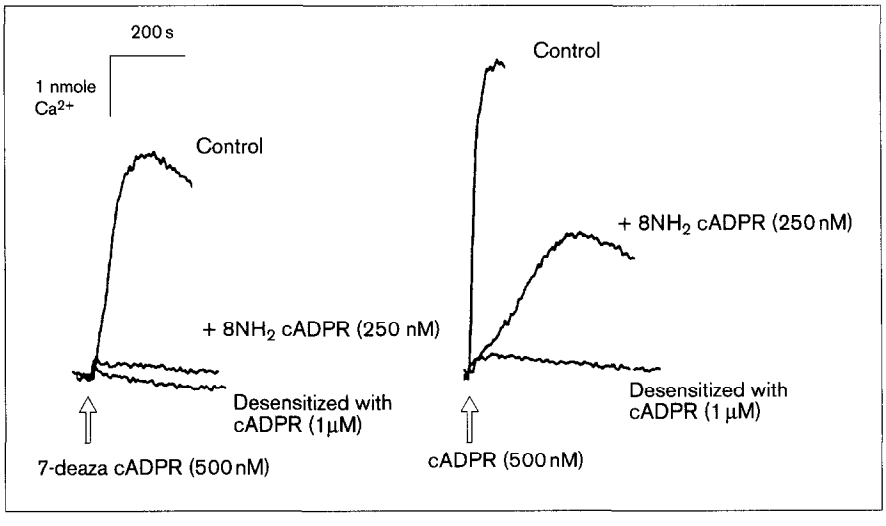
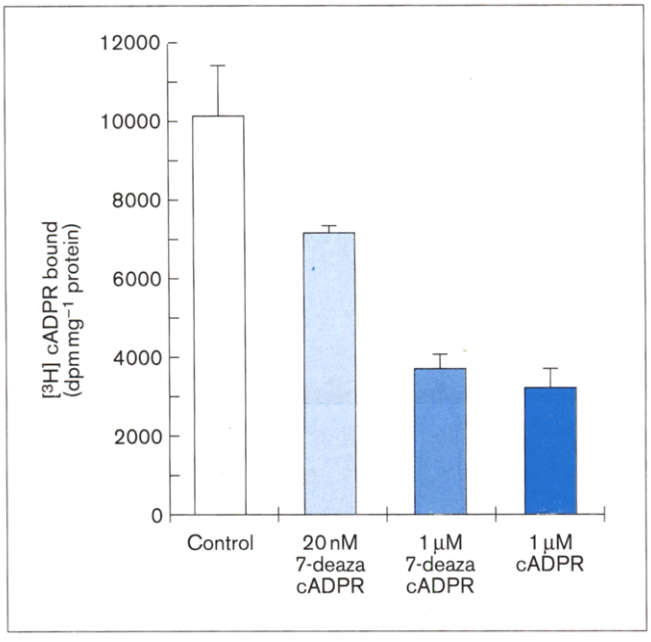


Figure 9



Action of 7-deaza cADPR on [^{3}H]-cADPR binding in sea urchin egg homogenates. [^{3}H]-cADPR binding was determined in sea urchin homogenates as described in the Materials and methods section. Specific cADPR binding was demonstrated by the amount of radiolabeled cADPR displaced by a maximal concentration of cADPR (1 μM); this was approximately 67% of total bound label. The same concentration of 7-deaza cADPR (1 μM) was equally effective at reducing radioligand binding. This result confirms that 7-deaza cADPR and cADPR interact with the same binding site. When radiolabeled cADPR (20 nM) was incubated with an equimolar concentration of 7-deaza cADPR, this resulted in approximately 50% displacement of specifically bound radioligand, suggesting that the binding affinities may be similar. Values represent mean ± SE of triplicate experiments.

adenine ring. The 7-deaza compound might be expected to present itself to the receptor in a similar way to cADPR, albeit now lacking one hydrogen-bond interaction and potentially interfering sterically with a putative hydrogen-bond donor. 7-Deaza cADPR is clearly nevertheless able to mimic cADPR sufficiently to induce substantial Ca^{2+} release. In the case of the antagonist 8-amino cADPR, however, the amino group might cause binding to the receptor in a way different to that of either 7-deaza cADPR or cADPR itself, thus shearing the molecule into an unfavourable conformation for the stimulation of ion-channel opening. These considerations must operate within the limitations of steric constraints at position 8 because it is known that bulky and hydrophobic substituents at position 8 lead to reduced and eventual loss of antagonist activity in sea urchin egg homogenate (our unpublished observations).

These results are also the first to demonstrate that resistance to hydrolytic stability in a cADPR analogue may also be engendered by a modification at position 7. Clearly, 7-deaza cADPR is not degraded as rapidly as is cADPR (Fig. 6), so it complements cArisDPR [17] in biological applications requiring metabolic stability.

Further work in this area is necessary before we understand the structural requirement for effective antagonism and in order to investigate the interplay of substituents at both position 7 and position 8. What is clear, however, is that this region of the imidazole ring is important in stimulating the Ca^{2+}-release machinery. In addition, such modification, perhaps in conjunction with that at position 8, should produce compounds of interesting biological activity. Thus, the results presented here further support the use and viability of the chemo-enzymatic approach for the production of pharmacologically important analogues of cADPR. They also provide a key new lead in the design of compounds to modulate the activity of cADPR. In addition, the data help to define how cADPR binding leads to the activation and opening of its target Ca^{2+}-release channel.

Significance

cADPR is a novel candidate second messenger whose properties are leading us to re-evaluate our understanding of cellular Ca^{2+} signaling. It has been shown to be active in a wide range of cell types in which its action is clearly of importance. A full understanding of its mechanism of action and interplay with other Ca^{2+}-release pathways is, therefore, of great pharmacological importance. At present there are very few molecular probes with which the action of cADPR can be investigated. By developing and exploiting a chemo-enzymatic synthetic approach, we have been able to synthesize analogues of cADPR from their parent nucleosides, which will allow a range of modified structures to be created. The only limiting factor is the substrate specificity of the cyclizing enzyme. Making small but significant changes in the structure of each new analogue can often result in dramatic differences in biological activity and so lead to the production of useful pharmacological tools.

In this paper, we describe the synthesis and evaluation of the first partial agonist of the cADPR Ca^{2+}-release mechanism. This compound is of significance by virtue of the demonstration that cADPR analogues of lower efficacy and with hydrolytic resistance can be obtained by modification at position 7 of the adenine ring. This points to a highly sensitive area around positions 7 and 8 of the imidazole ring. 7-Deaza cADPR is a key new lead compound; by further investigating the interplay of positions 7 and 8 together, we should be able to synthesize compounds with interesting biological activity and enhanced antagonist potential. Such tools will help us to understand how cADPR binding leads to Ca^{2+} release in this novel signaling pathway.

Materials and methods

Materials

7-Deaza adenosine (Tubercidin), nicotinamide mononucleotide (NMN), and 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulphonic acid (HEPES) were purchased from Sigma (London). ADP-ribosyl cyclase was isolated from *Aplysia californica* as described [27] and was used crude. cADPR and 8-amino cADPR were synthesized as described [16] and were purified by Q-Sepharose low-pressure ion-exchange chromatography and isolated as their triethylammonium salts. Fluo-3 dye was purchased from Calbiochem and fluorescence was monitored using a Perkin–Elmer LS-50B fluorimeter. NMR spectra were recorded on a Jeol FX-90Q, GX-270 or GX-400 spectrometer as indicated and chemical shifts were measured in parts per million (ppm) relative to the residual protonated solvent. ^{31}P spectra are also quoted in ppm relative to external 85% H_3PO_4; chemical shifts are positive when downfield from this reference. *J* values are given in Hz and the following abbreviations are used: s, singlet; d, doublet; t, triplet; m, multiplet. Mass spectra were recorded at the University of Bath Mass Spectrometry Service using positive and negative fast atom bombardment with *m*-nitrobenzyl alcohol as the matrix; the electrospray mass spectrum was recorded at the SERC Mass Spectra Service, University of Swansea. HPLC was performed on a Shimadzu LC-6A chromatograph with UV detection at 259 nm and using a 10 μm Partisil SAX anion exchange column isocratically eluted with 0.05 M KH_2PO_4/5% MeOH buffer. Ion-exchange chromatography was performed using a LKB Gradifrac system with the column packed with Q-Sepharose Fast Flow resin (Pharmacia) in the bicarbonate form. Gradient elution was performed using an ion-exchange buffer system of triethylammonium bicarbonate (TEAB) at pH 7.8. All other chemicals were purchased from Aldrich Chemical Company and solvents were distilled and dried using standard techniques.

*Preparation of 7-deaza adenosine 5′-monophosphate (**2**).*

7-Deaza adenosine was selectively phosphorylated at the 5′-hydroxyl using methods based on those described by Yoshikawa *et al.* [28] for the phosphorylation of nucleosides. A mixture of dry 7-deaza adenosine (82 mg, 308 μmol) and triethyl phosphate (1 ml) was heated at 50°C for 15 min. The suspension was cooled to 0°C and phosphorus oxychloride (50 μl, 535 μmol) was added drop-wise. A clear solution was seen after 5 min and this was stirred at 4°C for 18 h. Iced water (10 ml) was added and the mixture was stirred for a further 2 h. The product was purified by ion-exchange chromatography using a gradient of 0 to 300 mM TEAB over 720 ml and the nucleotide eluted between 190 mM and 220 mM. Appropriate fractions were pooled and evaporated *in vacuo* and excess TEAB was coevaporated with MeOH.

The nucleotide was further purified from contaminating inorganic phosphate by adsorption on to activated charcoal [29] by dissolving the product in water and stirring with the charcoal (1 g) for 15 min. The charcoal was filtered off, washed with water and the product was desorbed by elution with 25:24:1 (v\v\v) ethanol-water-concentrated ammonia (500 ml). The eluate was evaporated to dryness to give the title compound (**2**) as the ammonium salt in 48% yield as calculated by UV analysis. NMR: δ_H (270 MHz, D_2O) 3.94 (2H, m, H5′), 4.19 (1H, dt, *J*=3.1, 5.2 Hz, H4′), 4.30 (1H, dd, *J*=3.1, 5.0 Hz, H3′), 4.49 (1H, dd, *J*=5.0, 6.7 Hz, H2′), 6.14 (1H, d, *J*=6.7 Hz, H1′), 6.72 (1H, d, *J*=4.0 Hz, H7), 7.54 (1H, d, *J*=4.0 Hz, H8), 8.08 (1H, s, H2). δ_P (161.7 MHz, D_2O) 0.02 ppm (td, J_{PH}=2.4, 4.9 Hz). δ_C (100.4 MHz, D_2O) 67.3, 73.5, 77.1, 86.8, 89.2, 101.7, 105.5, 127.2, 145.3, 150.4, 153.9. UV: λ_{max}=266 nm, $\varepsilon=12.2\times10^3\,M^{-1}cm^{-1}$ [30]. HPLC single peak at 1.98 min (compared to adenosine 5′-monophosphate 2.15 min).

Preparation of 2′,3′-diacetyl NMN.

2′,3′-Diacetyl NMN was prepared from NMN essentially as described [31]. A solution of NMN, free acid, (15 mg, 50 μmol) in H_2O (0.1 ml) was added drop-wise to a rapidly stirred solution of acetic anhydride (0.78 ml) in dry pyridine (1.2 ml). The reaction mixture was stirred at room temperature for 2 h and then overnight at 4°C. The solvent was removed *in vacuo*, keeping the bath temperature at 30°C, and the residue was stirred with aqueous pyridine (1 ml) for 0.5 h to destroy any mixed anhydride and any excess of acetic anhydride. After removal of the solvent, the resulting residue was dissolved in MeOH (0.5 ml) and tri-*n*-octylamine (22 μl, 50 μmol) was added. The mixture was stirred for 0.5 h and the MeOH was evaporated. The residue was dried three times by coevaporation of any excess water with dry dimethyl formamide (DMF) and the resulting crude product was used directly in the next step.

*Preparation of 7-deaza NAD$^+$ (**3**)*

Coupling of the nucleotides was accomplished essentially using the Michelson procedure [19]. 7-Deaza adenosine 5′-monophosphate, free acid, (**2**, Fig. 3; 25 mg, 72 μmol) was dissolved in anhydrous MeOH (0.5 ml). To this was added one equivalent of tri-*n*-octylamine (32 μl) and the mixture was stirred until a clear solution was seen, (~0.5 h). The solvent was evaporated and the resulting mono-tri-*n*-octylammonium salt was dried by evaporation of three volumes of dry DMF (1 ml). The residue was dissolved in a mixture of dry DMF (0.3 ml) and dry dioxane (0.5 ml) and diphenyl chlorophosphate (DPPC, 20 μl, 100 μmol) was added, followed by tri-*n*-butylamine (20 μl, 75 μmol) . An homogeneous solution formed after a few minutes and this was further stirred at room temperature for 2 h. After this time a ^{31}P NMR spectrum (36.2 MHz, D_2O in a 10 mm outer tube) showed the formation of the activated nucleotide intermediate (**6**, Fig. 3) to be complete, with no peak remaining at approximately 0 ppm corresponding to 7-deaza adenosine 5′ monophosphate. The solvent was evaporated *in vacuo* and the residue was shaken with cold, dry ether (to remove any unreacted diphenyl chlorophosphate) to give a white solid. The majority of the ether was decanted and any residual ether was removed by evaporation.

Activated 7-deaza adenosine 5′-monophosphate (**6**, Fig. 3) was dissolved in dry DMF (0.2 ml). 2′,3′-Diacetyl NMN, used directly from the previous step, was also dissolved in DMF (0.3 ml). The solution was added to the activated nucleotide followed immediately by dry pyridine (0.5 ml) and the reaction was stirred at room temperature for 18 h. After this time, the solvent was evaporated and the product was deacetylated *in situ* by dissolving the residue in ice-cold methanolic ammonia (1:1 ratio of MeOH with 35% ammonia solution with a total volume of 1 ml) and stirring for 6 h at 4°C. The solvent was removed *in vacuo* and the residue was purified by ion-exchange chromatography using a gradient elution of 0 to 250 mM TEAB over 720 ml. The fractions containing the product, which eluted between 120 mM and 140 mM, were pooled, evaporated *in vacuo* and excess TEAB was coevaporated with MeOH. The title compound (**3**, Fig. 3) was obtained as the triethylammonium salt in 38% yield over the two steps, as quantified by UV measurements. NMR: δ_H (270 MHz, D_2O) 1.24 (9H, t, *J*=7.3 Hz, $N(CH_2CH_3)_3$), 3.17 (6H, q, *J*=7.3 Hz, $N(CH_2CH_3)_3$), 4.0–4.1 (4H, m, $2H_A5'$, $2H_N5'$), 4.19 (1H, m, H_A3'), 4.25 (1H, m, H_N3'), 4.29 (1H, m, H_A4'), 4.38 (1H, dt, *J*=5.3, 4.9 Hz, H_A2'), 4.42 (1H, m, H_N2'), 4.46 (1H, t, *J*=5.7 Hz, H_N4'), 6.11 (1H, d, *J*=5.3 Hz, H_A1'), 6.16 (1H, d, *J*=6.4 Hz, H_N1'), 6.65 (1H, d, *J*=3.6 Hz, H_A7), 7.53 (1H, d, *J*=3.6 Hz, H_A8), 8.09 (1H, s, H_A2), 8.16 (1H, dd, *J*=8.1, 6.4 Hz, H_N3), 8.78 (1H, d, *J*=8.1 Hz, H_N4), 9.15 (1H, d, *J*=6.4 Hz, H_N6), 9.33 (1H, s, H_N2). δ_P (161.7 MHz, D_2O) −11.8, −11.4 ppm (2d, J_{PP}=21.8 Hz). δ_C (100.4 MHz, D_2O) 9.1, 47.5, 65.8, 66.5, 71.3, 71.6, 75, 78.5, 84.5, 87.2, 87.9, 100.9, 103, 103.4, 124.6, 129.5, 134.4, 140.8, 143.3, 146.6, 149.1, 152.4, 155, 166.2. UV: λ_{max}=266 nm, $\varepsilon=12.7\times10^3\,M^{-1}cm^{-1}$ [32]. HPLC single peak at 2.19 min (compared to β-NAD$^+$ 2.48 min). m/z (electrospray) 663 (M^+), 662 (M^+–1), 661 (M^+–2), 540 (M^+-nicotinamide), 539 (M^+-nicotinamide-1).

*Synthesis of 7-deaza cADPR (**4**)*

7-Deaza NAD$^+$ (**3**, Fig. 3) was cyclized enzymatically using similar methods to those previously detailed [16]. A final volume of 2.5 ml of a 1.5 mM solution of 7-deaza NAD$^+$ in 25 mM HEPES buffer at pH 6.8 was incubated with 10 μl of crude *Aplysia* ADP-ribosyl cyclase at room temperature. The reaction was monitored by HPLC (Fig. 4) and was quenched by a 20-fold dilution of the reaction mixture with water when the peak attributable to the product was no longer seen to be increasing. The desired product was isolated from a small amount of

unreacted starting material and the by-product nicotinamide using ion-exchange chromatography eluting with 0 to 250 mM TEAB. The product, which eluted between 90 and 110 mM TEAB, was evaporated to dryness *in vacuo* keeping the water-bath temperature below 30°C. Excess TEAB was removed by evaporation with MeOH and the title compound (**4**) was isolated in 57% yield by UV as the glassy triethylammonium salt and stored at −70°C.

The product was quantified by total phosphate analysis using a modification of the Briggs test [33]. Three accurately known quantities of the sample were diluted in water (400 μl) and heated to dryness. The phosphate anhydride and resulting esters were hydrolyzed by addition of a few drops of conc. H_2SO_4 and by further heating at 175°C for 1.5 h. The samples were then cooled and the following solutions added: H_2O (200 μl); ammonium molybdate (400 μl; 12.5% w/v in water with 8 ml of conc H_2SO_4); hydroquinone (200 μl; 0.5% w/v in water with one drop of conc H_2SO_4) and sodium sulphite (200 μl; 20% w/v in water). Each solution was boiled vigorously for 10 s, transferred to a volumetric flask and made up to 5 ml with water. Each sample was repeated in triplicate and the UV absorbance was recorded at 340 nm. The amount of phosphate in each sample was calculated from a standard curve constructed using known amounts of KH_2PO_4 treated exactly as above and prepared immediately before use. This allowed the extinction coefficient to be calculated for the novel product. NMR δ_H (400 MHz, D_2O) 1.24 (3H, t, J=7.3 Hz, $N(CH_2CH_3)_3$), 3.17 (2H, q, J=7.3 Hz, $N(CH_2CH_3)_3$), 3.9–4.45 (6H, m, $2H_A5'$, $2H_R5'$, H_A4', H_R3'), 5.34 (1H, dd, J=5.6, 6.4 Hz, H_A2'), 5.75 (1H, d, J=6.4 Hz, H_A1'), 6.01 (1H, d, J=4.0 Hz, H_R1'), 6.71 (1H, d, J=3.7 Hz, H_A7), 7.31 (1H, d, J=3.7 Hz, H_A8), 8.75 (1H, s, H_A2); other protons are obscured by the water peak at 4.8 ppm. δ_P (161.7 MHz, D_2O) −12.7, −13.4 ppm (2d, J_{PP}=14.4 Hz). UV: λ_{max}= 272 nm, ε=8.15×10^3 M^{-1}cm^{-1}. HPLC: single peak at 4.87 min (compared to cADPR 4.20 min). m/z (FAB$^+$) 541 $(M+H)^+$, (FAB$^-$) 539 $(M-H)^-$.

Calcium-release measurements

Lytechinus pictus egg homogenate (2.5% v/v), prepared as previously described [20], was incubated at 17°C in intracellular-like medium (IM) containing an adenosine 5′-triphosphate-regenerating system, mitochondrial inhibitors and Fluo-3 (3 μM), and extra-microsomal Ca^{2+} was measured by monitoring Fluo-3 fluorescence (excitation 490 nm and emission 535 nm). Homogenate (0.5 ml) was placed in a cuvette and 5 μl additions of test compound (dissolved in IM with 10 μM EGTA) were made. Estimates of the amount of Ca^{2+} released were based on calibrations with known amounts of Ca^{2+} added to the assay homogenate, as described previously [20].

Determination of [^{3}H]-cADPR binding

[^{3}H]-cADPR binding was determined in sea urchin homogenates as described by Genazzani *et al.* [34]. In brief, homogenates were diluted in Glu-IM containing 1 mM EGTA to a concentration of 1 mg ml^{-1} protein (~3.5%) and incubated with 20 nM [^{3}H]-cADPR for 10 min at 4°C. Non-specific binding was assessed with 1 μM cADPR. Binding was terminated by filtration (fiberglass GF/B filters) under vacuum and filters were rapidly washed twice with ice-cold Glu-IM. Filters were dissolved in 14 ml scintillation fluid and radioactivity was determined by liquid scintillation counting.

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