Roles for Adenosine Ribose Hydroxyl Groups in Cyclic Adenosine 5′-Diphosphate Ribose-Mediated Ca^{2+} Release†

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ABSTRACT: Cyclic adenosine diphosphate ribose (cADPR) is a naturally occurring and potent Ca^{2+}-mobilizing agent. Structural analogues are currently required as pharmacological tools for the investigation of this topical molecule, but modifications to date have concentrated primarily upon the purine ring. Two novel dehydroxylated analogues of cADPR have now been prepared from chemically synthesized nicotinamide adenine dinucleotide (NAD^+) precursors modified in the ribose moiety linked to adenine. ADP-ribosyl cyclase of *Aplysia californica* catalyzed the conversion of $2'_A$-deoxy-NAD^+ and $3'_A$-deoxy-NAD^+ into the corresponding $2'_A$-deoxy-cADPR and $3'_A$-deoxy-cADPR analogues, respectively. These analogues were used to assess the effect of 2′- and 3′-hydroxyl group deletion in the adenosine ribose moiety of cADPR on the Ca^{2+}-releasing potential of cADPR. These compounds were found to have comparatively markedly different activities as agonists for Ca^{2+} mobilization in sea urchin egg homogenate. $2'_A$-Deoxy-cADPR is similar to cADPR, whereas $3'_A$-deoxy-cADPR is at least 100-fold less potent, indicating that the $3'_A$-hydroxyl group, but not the $2'_A$-hydroxyl group, is essential for calcium releasing activity. EC_{50} values recorded were 32 nM, 58 nM, and 5 μM for cADPR, $2'_A$-deoxy-cADPR, and $3'_A$-deoxy-cADPR, respectively. Moreover, 200 nM $2'_A$-deoxy-cADPR was required to desensitize the cADPR-sensitive Ca^{2+} channel to a subsequent addition of 100 nM cADPR, but 20 μM $3'_A$-deoxy-cADPR was required to produce the same desensitizing effect. This is in accordance with the 100-fold lower potency exhibited by the latter analogue. To further investigate the importance of the 3′-hydroxyl group, we have also synthesized $3'_A$-*O*-methyl-cADPR, in which the 3′-hydroxyl group of adenosine has been methylated and its ability potentially to donate a hydrogen atom in a hydrogen bond has been removed. Although inactive in releasing Ca^{2+}, $3'_A$-*O*-methyl-cADPR inhibited cADPR-induced Ca^{2+} release in a dose-dependent manner with an approximate IC_{50} value of 5 μM, whereas 3′-*O*-methyladenosine had no effect. This further supports the requirement of a 3′-OH group for Ca^{2+} releasing activity. In addition, however, it suggests that this group may not be crucial for ligand−receptor recognition. Thus, replacement of the hydrogen atom of the hydroxyl with a methyl group effects a change of activity from an agonist to an antagonist of cADPR-induced Ca^{2+} release. Two other analogues with modifications in the 2′ and/or 3′ positions, 3′-cADPR phosphate and 2′,3′-cyclic-cADPR phosphate, were synthesized and tested for their Ca^{2+}-mobilizing activity in sea urchin egg homogenates. Both analogues were inactive with respect to both agonistic and antagonistic activities on the cADPR-sensitive Ca^{2+} release mechanism. These are the first steps toward a wider structure−activity relationship for cADPR, and this is the first study to implicate a crucial role for the adenosine ribose hydroxyl groups of cADPR in the biological activity of this cyclic nucleotide. Additionally, this is the first report of a cADPR receptor antagonist that is not modified at the 8-position of the purine ring.

Cyclic adenosine diphosphate ribose (cADPR;[1] structure **1**, Figure 1), a metabolite of NAD^+, was first discovered during investigations of the Ins(1,4,5)P_3-independent Ca^{2+} mobilization mechanisms in sea urchin egg preparations (Clapper et al., 1987; Dargie et al., 1989). It has been suggested to be the physiological modulator of the ryanodine receptor (Galione et al., 1993a), although there is increasing evidence that cADPR may not act directly on the ryanodine channel but that its agonistic action is mediated by an accessory protein (Walseth et al., 1993), which has recently been suggested to be calmodulin (Lee et al., 1994a). Current evidence, therefore, is indicative of a binding site distinct from that of ryanodine. cADPR releases Ca^{2+} by a pathway independent of D-*myo*-inositol 1,4,5-trisphosphate [Ins-(1,4,5)P_3]. The widespread occurrence of cADPR in mammalian cells and the ubiquitous presence of the enzymes that

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[1] Abbreviations: cADPR, cyclic adenosine 5′-diphosphate ribose; NAD^+, nicotinamide adenine dinucleotide; DCC, *N,N*-dicyclohexylcarbodiimide; DCU, dicyclohexylurea; AMP, adenosine 5′-monophosphate; KH_2PO_4, potassium dihydrogen phosphate; H_3PO_4, orthophosphoric acid; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; R_t, high-performance liquid chromatography retention time; $PO(OEt)_3$, triethyl phosphate; $POCl_3$, phosphorus oxychloride; RFU, relative fluorescence units; TEAB, triethylammonium bicarbonate; ES, electrospray.

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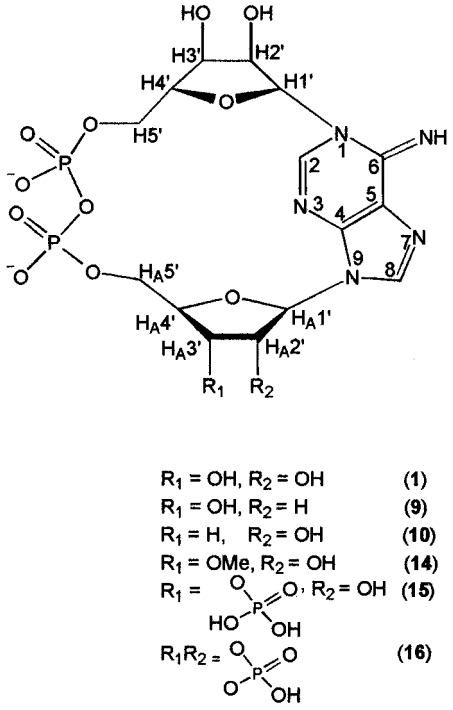

FIGURE 1: Structures of cADPR (**1**), $2'_A$-deoxy-cADPR (**9**), $3'_A$-deoxy-cADPR (**10**), $3'_A$-OMe-cADPR (**14**), $3'_A$-P-cADPR (**15**), and $2'_A,3'_A$-cyclic-P-cADPR (**16**).

synthesize and hydrolyze it indicate the generality of this Ca^{2+} release system (Rusinko et al., 1989; Galione & Sethi, 1996). A plethora of mammalian tissues have also been shown to be responsive to cADPR, e.g., cardiac myocytes (Rakovic et al., 1996), pituitary cells (Koshiyama et al., 1991), and pancreatic β-cells (Takesawa et al., 1993) among others. The presence of endogenous cADPR and its ability to mobilize Ca^{2+} in T-lymphocyte cell lines (Guse et al., 1995) and mouse T-lymphoma cells (Bourguignon et al., 1995) has also recently been demonstrated. Cholecystokinin octapeptide has been reported to augment the synthesis of cADPR in mammalian intestinal longitudinal smooth muscle in a concentration-dependent fashion (Kuemmerle & Makhlouf, 1995).

cADPR (**1**) is a cyclized ADP-ribose having an N-ribosyl linkage between the anomeric carbon C1′ and the N1 of the adenine ring (Figure 1).[2] The N-ribosyl linkage was originally thought to be at N6, but UV data (Kim et al., 1993), together with the crystal structure of cADPR (Lee et al., 1994b) and various NMR data (Wada et al., 1995), have confirmed that the position of cyclization is between the anomeric carbon of the terminal ribose C1′ and the N1 of adenine and that the linkage is of the β-configuration.

Endogenous cADPR is synthesized from NAD^+ (**2**) by ADP-ribosyl cyclase (Lee & Aarhus, 1991; Glick et al., 1991), with the concomitant release of nicotinamide. It is

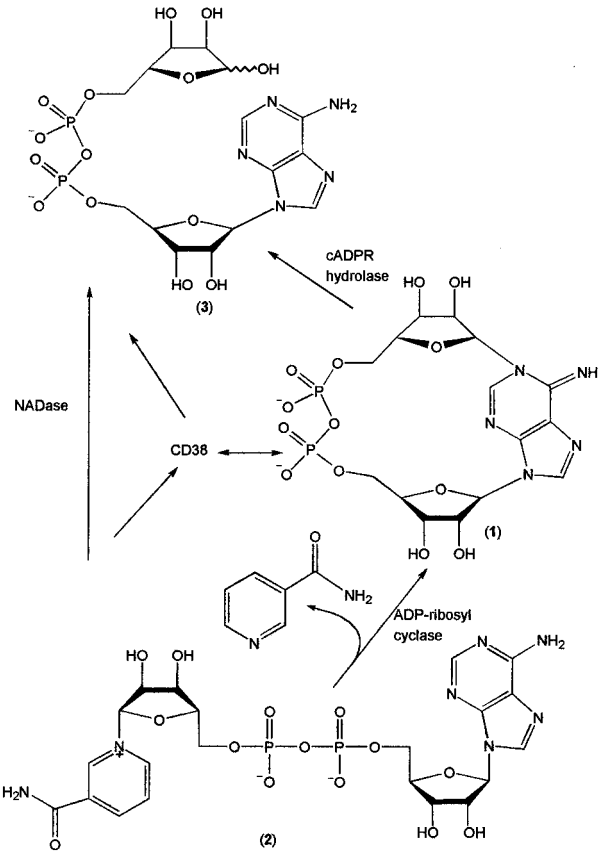

FIGURE 2: Formation and metabolism of cADPR (**1**). cADPR is synthesized from NAD^+ by ADP-ribosyl cyclase and hydrolyzed to its inactive metabolite ADP-ribose by cADPR hydrolase. CD38 is a bifunctional enzyme that catalyzes both the synthesis and degradation of cADPR.

degraded by cyclic ADP-ribose hydrolase to ADP-ribose (**3**). Both the substrate (NAD^+) and products have no Ca^{2+}-mobilizing activity (Galione & Sethi, 1996; Lee & Aarhus, 1993). The synthesis and metabolism of cADPR is shown in Figure 2. A lymphocyte differentiating cell surface antigen CD38 (Howard et al., 1993; Summerhill et al., 1993) has also been shown to catalyze both the formation and hydrolysis of cADPR. Recent work has demonstrated that invertebrate ADP-ribosyl cyclase and CD38 may be crucial enzymes in Ca^{2+} signaling, as these enzymes catalyze the conversion of NAD^+ and $NADP^+$ to cADPR and $NAADP^+$, respectively. These two different products are involved in Ca^{2+} mobilization from Ins(1,4,5)P_3 and cADPR-insensitive Ca^{2+} stores in sea urchin egg homogenates (Lee & Aarhus, 1995; Aarhus et al., 1995; Genazzani & Galione, 1996). ADP-ribosyl cyclase has also been shown to mediate both nitric oxide- and cGMP-induced Ca^{2+} release (Galione et al., 1993b; Willmott et al., 1995; Sethi et al., 1996), thus further implicating it as a putative second messenger-synthesizing enzyme involved in Ca^{2+} regulation.

cADPR has recently been synthesized by chemical cyclization of NAD^+ (Yamada et al., 1994) and also through enzymatic cyclization of N^1-(5′-phosphoribosyl)ATP catalyzed by NAD^+ pyrophosphorylase (Gu & Sih, 1994). However, the yields of these approaches are poor and the methods are not general. We believe that the most versatile route to chemically modified analogues as potential novel agonists, antagonists, and enzyme inhibitors is via enzymatic cyclization of NAD^+ analogues catalyzed by ADP-ribosyl cyclase. We and others have demonstrated the broad

[2] Note that in the nomenclature used in this paper, H_N1', -2′, -3′, -4′, and -5′ refer to protons on the nicotinamide ribose; H_N2, -4, -5, and -6 refer to protons on the nicotinamide ring; and H_A1', -2′, -3′, -4′, and -5′ refer to protons on the adenine ribose in NAD^+ and NAD^+ analogues as shown in Figure 3. In cADPR and analogues, H1′, -2′, -3′, -4′, and -5′ refer to protons on the ribose that previously carried the nicotinamide moiety in the NAD^+ precursor and H_A1', -2′, -3′, -4′, and -5′ refer to protons on the other ribose ring. C1′ refers to the carbon atom to which H1′ is attached. N1 and N6 refer to the nitrogen atom at positions 1 and 6 on the adenine ring, respectively, as shown in Figure 1.

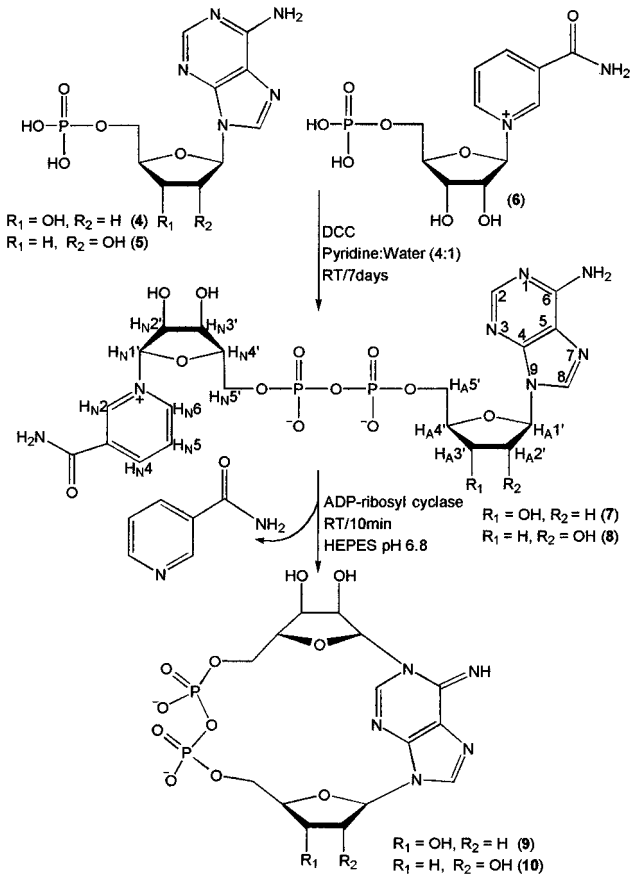

FIGURE 3: Schematic representation of the chemoenzymatic synthesis of 2′$_A$-deoxy-cADPR and 3′$_A$-deoxy-cADPR. 2′$_A$-Deoxy-NAD$^+$ (**7**) and 3′$_A$-deoxy-NAD$^+$ (**8**) were synthesized chemically by coupling of NMN (**6**) to 2′$_A$-deoxy-AMP (**4**) and 3′$_A$-deoxy-AMP (**5**), respectively, followed by enzymatic cyclization to the corresponding cyclized structures 2′$_A$-deoxy-cADPR (**9**) and 3′$_A$-deoxy-cADPR (**10**), respectively.

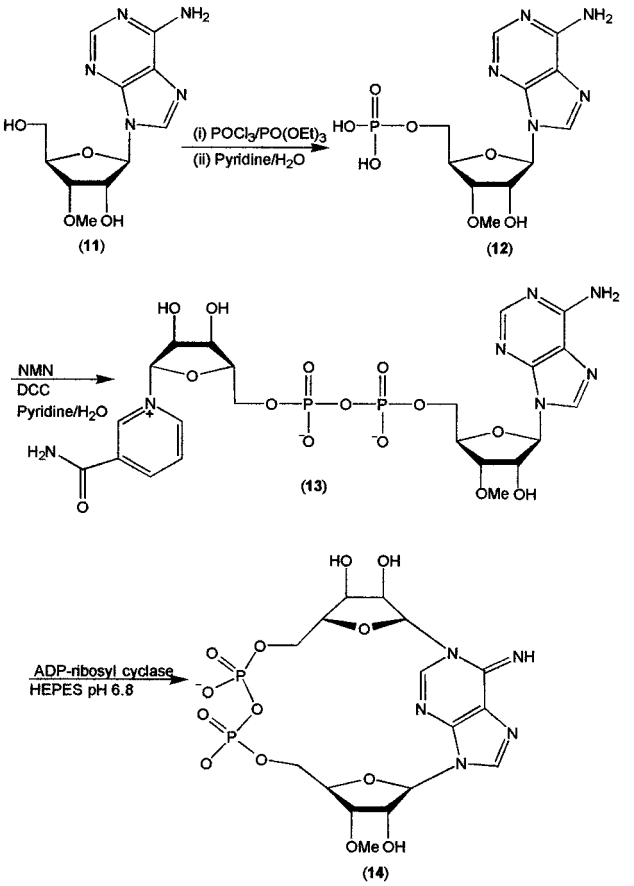

FIGURE 4: Schematic representation of the synthesis of 3′$_A$-OMe-cADPR. 3′$_A$-OMe-adenosine (**11**) was selectively phosphorylated to 3′$_A$-OMe-AMP (**12**), followed by chemical coupling to NMN to give 3′$_A$-OMe-NAD$^+$ (**13**). Enzymatic cyclization of 3′$_A$-OMe-NAD$^+$ yielded 3′$_A$-OMe-cADPR (**14**).

substrate specificity for this enzyme (Ashamu et al., 1995; Walseth & Lee, 1993). Enzymatic conversion of NAD$^+$ into cADPR was first demonstrated by using crude sea urchin egg extracts (Clapper et al., 1987). Extracts from both *Lytechinus pictus* and *Stronglyocentrotus purpuratus* were both active in producing cADPR. Routine use of *Aplysia* cyclase is favorable over sea urchin egg cyclase due to the higher ADP-ribosyl cyclase activity and minimal (if any) hydrolase activity and the unusual abundance of this enzyme in *Aplysia californica*.

Our current interest focuses upon identifying the key recognition elements of cADPR that contribute to its potent Ca^{2+} releasing activity and also those important structural elements of NAD$^+$ that are recognized by ADP ribosyl cyclase. Our approach in understanding the structure−activity relationship of cADPR relies primarily upon synthesis of chemically modified NAD$^+$ analogues. We report here a chemoenzymatic synthesis of two dehydroxylated analogues of cADPR, 2′$_A$-deoxy- (**9**) and 3′$_A$-deoxy-cADPR (**10**), from their respective deoxy-NAD$^+$ analogues **7** and **8** (Figures 1 and 3). A preliminary account of some of this synthetic work has already been reported (Ashamu et al., 1995). In addition, three other novel cADPR analogues with modifications in the 2′$_A$ and or 3′$_A$ positions have also been synthesized (**14**, **15**, and **16**). 3′$_A$-*O*-methyl-cADPR (**14**) was prepared via chemical synthesis of 3′$_A$-*O*-methyl-NAD$^+$ (**13**), from 3′$_A$-*O*-methyl-adenosine (**11**) (Figure 4) followed by enzymatic cyclization to the corresponding cyclic nucleotide. 3′$_A$-cADPR phosphate (**15**) (3′$_A$-P-cADPR) and 2′$_A$,3′$_A$-cyclic-cADPR phosphate (**16**) (2′$_A$3′$_A$-cyclic-P-cADPR) were also synthesized. We report here an evaluation of the Ca^{2+}-mobilizing activity of these cADPR analogues and the implications for structure−activity relationships.

EXPERIMENTAL PROCEDURES

Materials and Methods. Nicotinamide 5′-mononucleotide (NMN), 2′$_A$-deoxyadenosine 5′-monophosphate (2′$_A$-deoxy-AMP), 3′$_A$-deoxyadenosine 5′-monophosphate (3′$_A$-deoxy-AMP), 3′$_A$-*O*-methyladenosine (3′$_A$-OMe-adenosine), 3′$_A$-nicotinamide adenine dinucleotide (3′$_A$-NADP), 2′$_A$, 3′$_A$-cyclic nicotinamide adenine dinucleotide (2′$_A$,3′$_A$-NADP), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane sulfonic acid (HEPES), *N*,*N*-dicyclohexylcarbodiimide (DCC), NAD$^+$, yeast alcohol dehydrogenase, and tris(hydroxymethyl)-aminomethane (Tris) were purchased from Sigma (London). Organic solvents and other reagents were obtained from Aldrich. Pyridine was dried overnight with calcium hydride, redistilled, and stored over potassium hydroxide pellets. *Aplysia* ovotestis extracts containing ADP-ribosyl cyclase were prepared as previously described (Lee & Aarhus, 1991) and were used crude. The final protein concentration of the *Aplysia* cyclase preparation used in all cases was estimated as 10 mg/mL total protein using a Bio-Rad protein estimation kit. Fluo-3 was purchased from Molecular Probes Inc.

(Cambridge BioScience). All other reagents were from Sigma (London). Ion-exchange chromatography was performed on an LKB-Pharmacia medium-pressure ion-exchange chromatograph using a Sepharose Q fast flow column with gradients of triethylammonium bicarbonate buffer (TEAB), pH 7.6, as eluent. TEAB (1 M) was prepared by bubbling carbon dioxide gas into 1 M triethylamine solution for ca. 6 h. HPLC was performed on a Shimadzu LC-6A chromatograph with the UV detector operating at 259 nm using a combination of a Partisil 10 μm SAX guard column (10 × 0.46 cm) and a Technicol (10 × 0.46 cm) 10 μm SAX HPLC column or using a Spherisorb 10 μm SAX (25 × 0.46 cm) column with an isocratic elution using phosphate buffer (KH_2PO_4), pH 3.0, at a flow rate of 1 mL/min. ^{1}H NMR and ^{31}P NMR spectra were recorded on either Jeol JNM GX-270 FT NMR or Jeol EX-400 FT NMR spectrometers. Chemical shifts were measured in parts per million relative to deuteriated water (D_2O) for ^{1}H NMR and to external 85% H_3PO_4 for ^{31}P NMR. For the latter, δ values are positive when downfield from this reference. *J* values are given in Hertz (Hz). Mass spectra were recorded at the EPSRC Mass Spectrometry Service Center at the University of Swansea and at the University of Bath. Ultraviolet (UV) absorbance was measured with a Perkin-Elmer Lambda 3 UV/vis spectrophotometer.

Chemical Synthesis of NAD$^+$ Analogues: (A) 2′$_A$-Deoxy-NAD$^+$ (7). NMN (**6**) (50 mg, 149.6 μmol) and 2′$_A$-deoxy AMP (**4**) (80 mg, 241.5 μmol) were dissolved in 12.5 mL of 75% (v/v) pyridine/water. *N*,*N*-Dicyclohexylcarbodiimide (DCC) (2 g, 9.7 mmol) was added to the mixture and the solution was left stirring at room temperature for 7 days (Hughes et al., 1957; Fawcett & Kaplan, 1961) (Figure 3). The reaction was quenched by pouring the mixture into 150 mL of cold water, and the suspension was left at 0 °C for 2 h to allow the DCU to precipitate. DCU was filtered off through a sintered glass funnel, and the filtrate was extracted with 3 × 50 mL aliquots of chloroform. The aqueous layer was evaporated to dryness *in vacuo*, and the residue was redissolved in MilliQ water and purified on a Sepharose Q ion-exchange column, using a gradient of 0−500 mM TEAB buffer, pH 7.6. 2′$_A$-Deoxy-NAD$^+$ eluted as the triethylammonium (TEA) salt between 160 and 180 mM TEAB, in 19% yield, and was quantified by measuring UV absorbance at 259 nm, using an extinction coefficient of 17 800 M^{-1} cm^{-1}.

^{1}H NMR (D_2O, 270 MHz): δ 9.3 (H_N2,s), 9.1 (H_N6, d, J = 6.2 Hz), 8.78 (H_N4, d, J = 8 Hz), 8.3 (H_A8, s), 8.1 (H_N5, t, J = 7 Hz), 8.0 (H_A2, s), 6.36 (H_A1′, t, J = 7 Hz), 6.02 (H_N1′, d, J = 5.5 Hz), 4.7−4.2 (9 ribose protons, H_A3′, −4′, −5′, and −5′ and H_N2′, −3′, −4′ −5′, and −5′), 2.8 (H_A2′, m), and 2.5 (H_A2′, m). ^{31}P NMR (D_2O, 270 MHz): δ −11.08 and −10.72 (2 P, J_{PP} = 20 Hz). *m/z* (+ve ion FAB) 648 (M + H)$^+$; *m/z* (−ve ion FAB) 647 (M)$^-$ and 524 [M − H − nicotinamide)]$^-$. λ_{max}(pH 8.3) 259 nm, ϵ_{259} = 17 800 M^{-1} cm^{-1}.

(B) 3′$_A$-Deoxy-NAD$^+$ (8). NMN (15.7 mg, 47.4 μmol) and 3′$_A$-deoxy-AMP (**5**) (25 mg, 75.4 μmol) were dissolved in 6 mL of 75% aqueous pyridine. DCC (0.63 g, 3.05 mmol) was added to the solution, which was left stirring at room temperature for 7 days. The reaction was poured into 50 mL of cold distilled water and the mixture was left to stand for 2 h at 0 °C, after which the precipitate formed was filtered off. Water-insoluble impurities were extracted by shaking the filtrate with 3 × 50 mL aliquots of chloroform. The aqueous layer was dried *in vacuo*, and the residue was redissolved in MilliQ water and purified by ion-exchange chromatography, using a buffer gradient of 0−300 mM TEAB, pH 7.6. 3′$_A$-Deoxy-NAD$^+$ eluted between 165 and 185 mM TEAB, in 25% yield, and was quantified by measuring UV absorbance at 259 nm using an extinction coefficient of 17 800 M^{-1} cm^{-1}.

^{1}H NMR (D_2O, 270 MHz): δ 9.2 (H_N2, s), 9.0 (H_N6, d, J = 6.2 Hz), 8.7 (H_N4, d, J = 7.7 Hz), 8.2 (H_A8, s), 8.1 (H_N5, t, J = 7.5 Hz), 8.0 (H_A2, s), 6.0 (H_N1′, d, J = 6.0 Hz), 5.9 (H_A1′, d, J = 3 Hz), 4.7−4.0 (9 H), 2.3 (H_A3′, m), and 2.1 (H_A3′, m). ^{31}P NMR (D_2O, 270 MHz): δ −10.26 and −10.74 (2 P, J_{PP} = 20 Hz). MS: *m/z* (+ve ion FAB) 648 (M + H)$^+$; *m/z* (−ve ion FAB) 647 (M)$^-$, 524 [M − H − nicotinamide)]$^-$. λ_{max}(pH 8.3) 259 nm, ϵ_{259} = 17 800 M^{-1} cm^{-1}.

(C) 3′$_A$-OMe-NAD$^+$ (13). 3′$_A$-OMe-NAD$^+$ was synthesized from 3′$_A$-OMe-adenosine (**11**) using a two-step procedure. 3′$_A$-OMe-Adenosine was selectively phosphorylated at the 5′ position to give 3′$_A$-OMe-AMP (**12**) using a general procedure reported for nucleosides (Yoshikawa et al., 1967). 3′$_A$-OMe-Adenosine (100 mg, 0.355 mmol) was dissolved in 2 mL of $PO(OEt)_3$ and heated for a few seconds. The resulting solution was cooled to 0 °C and 50 μL of $POCl_3$ was added dropwise while the temperature was maintained at 0 °C. The reaction mixture was left stirring for 2 h at room temperature under anhydrous conditions, after which the reaction was quenched by stirring in 4 mL of pyridine: water (1:3) for 30 min. The solvent was removed *in vacuo* and excess $PO(OEt)_3$ was extracted into ice cold petroleum ether 40−60 °C. The resulting monophosphate (3′$_A$-OMe-AMP) was purified by ion-exchange chromatography. 3′$_A$-OMe-AMP was obtained in 48% yield (0.17 mmol) and was then coupled to NMN to obtain 3′$_A$-OMe-NAD$^+$ as follows. NMN (**6**) (149 μmol) and 3′$_A$-OMe-AMP (**12**) (170 μmol) were dissolved in 8 mL of 75% aqueous pyridine. DCC (2.0 g, 9.5 mmol) was added to the solution, which was left stirring at room temperature for 7 days. The reaction was poured into 50 mL of cold distilled water and the mixture was left to stand for 2 h at 0 °C, after which the precipitate formed was filtered off. Water-insoluble impurities were extracted by shaking the filtrate with 3 × 50 mL aliquots of chloroform. The aqueous layer was dried *in vacuo* and the residue was redissolved in MilliQ water and purified by ion-exchange chromatography, using a buffer gradient of 0−120 mM TEAB, pH 7.6. 3′$_A$-OMe-NAD$^+$ coeluted with NMN between 40 and 55 mM buffer. There was no improvement in purity by using a shallow gradient of TEAB from 0 to 70 mM. Contaminating excess NMN was removed from the desired product by treating the mixture with alkaline phosphatase for 4 h. Care was taken not to leave the solution for too long to avoid degradation of 3$_A$′-OMe-NAD$^+$. After 4 h most of the excess NMN had been converted to nicotinamide mononucleoside, which eluted straight off the ion-exchange column during purification of **13**. The sample of 3′$_A$-OMe-NAD$^+$ thus obtained was 83% pure by HPLC. This material was used directly to obtain the cyclized product, as further attempts to purify it would have resulted in more material loss.

3′$_A$-OMe-AMP: ^{1}H NMR (D_2O, 400 MHz) δ 8.3 (H_A8), 7.9 (H_A2, s), 5.9 (H_A1′, d, J = 5.8 Hz), 4.75 (H_A2′, dd, J = 5.5 and 5.8 Hz), 4.3 (H_A3′, t), 4.05 (H_A4′, m), 3.9 (H_A5′,5′,

m), and 3.4 (3H, Me, s); ^{31}P NMR (D_2O, 400 MHz) δ 2.3, s, 1 P (^{1}H-decoupled); *m/z* (ES) 360 [100%, $(M - H)^-$]; λ_{max} (pH 8.3) 259 nm $\epsilon_{259} = 15\,300\ M^{-1}\ cm^{-1}$.

3′$_A$-OMe-NAD$^+$: ^{1}H NMR (D_2O, 270 MHz) δ 9.4 (H_N2, s), 9.2 (H_N6, d, $J = 6.1$ Hz), 8.7 (H_N4, d, $J = 7.3$ Hz), 8.5 (H_A8, s), 8.35 (H_A2, s), 8.2 (H_N5, t), 6.15 (H_N1′, d, $J = 6.5$ Hz), 5.9 (H_A1′, d, $J = 5.2$ Hz), 4.8 (H_A2′, dd, $J = 5.2$ and 5.5 Hz), 4.6−4.0 (9 H), and 3.5 (3 H, s, -OMe); ^{31}P NMR (D_2O, 400 MHz) δ −11.5 and −11.8 (2 P, $J_{PP} = 19.8$ Hz); λ_{max}(pH 8.3) 256 nm.

Qualitative Analysis of NAD$^+$ Analogues. The authenticity of NAD$^+$ analogues thus synthesized was confirmed by testing for coenzyme activity (Suhaldonik et al., 1977) and the ability to form a complex with cyanide. Conversion of these NAD$^+$ analogues into the reduced form NADH by incubation with yeast alcohol dehydrogenase in the presence of 5% v/v ethanol in 0.1 M Tris buffer, pH 9, was monitored by UV analysis. A new peak at 340 nm was recorded as a result of this conversion. Complex formation with cyanide was performed as described previously (Colowick et al., 1951). The reaction was also monitored by UV and the appearance of the new cyanide adduct was recorded at 325 nm.

*Enzymatic Cyclization of NAD$^+$ and NAD$^+$ Analogues: (A) Synthesis of cADPR (**1**).* NAD$^+$ (2.5 mL of 1.5 mM solution) in 25 mM HEPES, pH 6.8, was incubated with 10 μL of crude ADP-ribosyl cyclase for 10 min at room temperature. The sample was analyzed before and after enzymatic incubation by HPLC using a Partisil 10 μm SAX guard column (10 × 0.46 cm) in combination with a Technicol (10 × 0.46 cm) SAX column and isocratic elution with 0.05 M phosphate buffer, pH 3. After 10 min, the incubation mixture was diluted with 100 mL of MilliQ water and the products were purified by ion-exchange chromatography using a gradient of 0−300 mM TEAB, pH 7.6. The purity of the product obtained was verified by HPLC analysis as described above. cADPR was obtained in 60% yield and was quantified by measuring UV absorbance at 254 nm using an extinction coefficient of 14 300 $M^{-1}\ cm^{-1}$ (Rusinko & Lee, 1989).

R_t (incubation mixture after 10 min) 4.7 (nicotinamide), 7.8 (cADPR), and 10.3 min (NAD$^+$). ^{1}H NMR (D_2O, 400 MHz): δ 8.9 (H_A2, s), 8.2 (H_A8, s), 6.0 (H1′, d, $J = 3.8$ Hz), 5.85 (H_A1′, d, $J = 7$ Hz), 5.2 (H_A2′, t, $J = 7$ Hz), 4.7 (H_A3′), 4.6 (H2′, H4′), 4.4 (H3′, H_A5′), 4.3 (H5′), 4.2 (H_A4′), 4.0 (H5′), and 3.8 (H_A5′). ^{31}P (D_2O, 400 MHz): δ −11.36 and −11.84 (2 P, $J_{PP} = 14.9$ Hz). MS: *m/z* (+ve ion FAB) 541 $(M)^+$, *m/z* (−ve ion FAB) 540 $(M - H)^-$.

*(B) Synthesis of 2′$_A$-Deoxy-cADPR (**9**).* Enzymatic cyclization of 2′$_A$-deoxy-NAD$^+$ was carried out as described above for NAD$^+$. HPLC analysis was on a Spherisorb SAX column using isocratic elution with phosphate buffer (typical chromatograms are shown in Figure 5). Ion-exchange purification of the product was carried out using a gradient of 0−300 mM TEAB, pH 7.6. Nicotinamide eluted straight off the column, followed by 2′$_A$-deoxy-cADPR between 190 and 240 mM TEAB, in 61% yield, and finally 2′$_A$-deoxy-ADPR. Pure **9** was quantified by measuring UV absorbance at 254 nm using an extinction coefficient of 14 300 $M^{-1}\ cm^{-1}$.

R_t (incubation mixture, Figure 4) 3.1 (nicotinamide), 4.9 (2′$_A$-deoxy-NAD$^+$), 7.2 (2′$_A$-deoxy-cADPR), and 10.2 min (2′$_A$-deoxy-ADPR). ^{1}H NMR (D_2O, 400 MHz): δ 8.9 (H_A2, s), 8.2 (H_A8, s), 6.4 (H_A1′, t, $J = 7$ Hz), 6.1 (H1′, d, $J = 5.5$ Hz), 4.8 (H_A3′), 4.7 (H2′, t), 4.6 (H4′), 4.4 (H3′, H_A5′), 4.3 (H_A5′), 4.2 (H_A4′), 4.0(H5′), 3.9(H_A5′), 3.2 (H_A2′, m), and 2.4 (H_A2′, m). ^{31}P NMR (D_2O, 400 MHz): δ −11.37 and −11.68 (2 P, $J_{PP} = 15.8$ Hz) (Figure 6). MS: *m/z* (+ve ion FAB) 1051 $(2M + H)^+$ dimer and 627 $(M + TEA)^+$; *m/z* (−ve ion FAB) 524 $(M - H)^-$ 1049 $(2M - H)^-$. λ_{max}(pH 8.3) 259 nm.

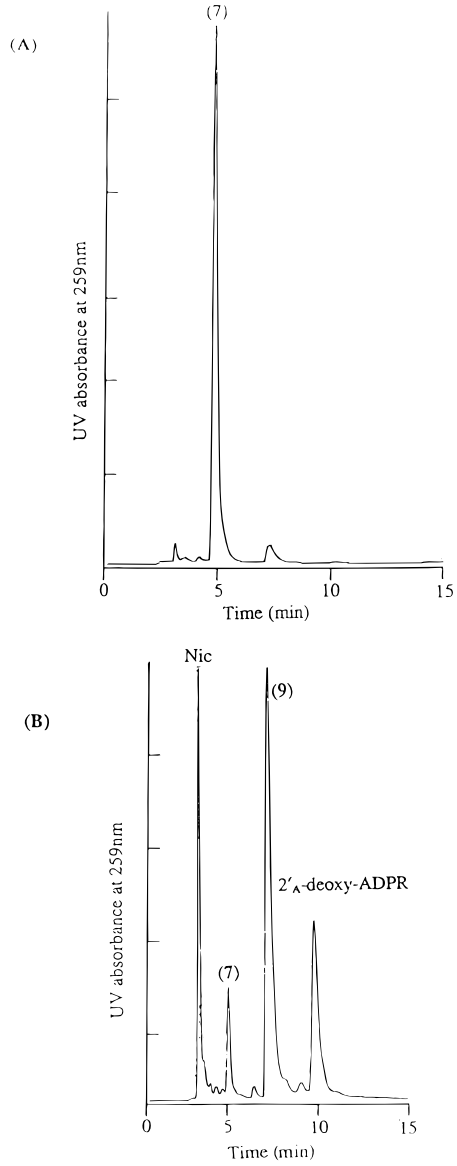

FIGURE 5: HPLC analysis of the cyclization of 2′$_A$-deoxy-NAD$^+$. Conversion of 2′$_A$-deoxy-NAD$^+$ (**7**) to 2′$_A$-deoxy-cADPR was monitored by HPLC on an anion-exchange column described in the text. (A) 2′$_A$-deoxy-NAD$^+$ as the starting material; (B) products obtained 10 min after incubation with crude ADP-ribosyl cyclase—nicotinamide (Nic), 2′$_A$-deoxy-cADPR (**9**), and 2′$_A$-deoxy-ADPR.

*(C) Synthesis of 3′$_A$-Deoxy-cADPR (**10**).* Enzymatic cyclization of 3′$_A$-deoxy-NAD$^+$, purification, and the analytical procedures used were as described for NAD$^+$ above. 3′$_A$-Deoxy-cADPR eluted from the ion-exchange column between 165 and 185 mM TEAB in 50% yield and was quantified by measuring UV absorbance at 254 nm using an extinction coefficient of 14 300 $M^{-1}\ cm^{-1}$.

R_t (incubation mixture after 10 min) 4.85 (nicotinamide), 11.3 (3′$_A$-deoxy-cADPR), and 18.3 min (3′$_A$-deoxy-NAD$^+$). ^{1}H NMR (D_2O, 400 MHz): δ 8.7 (H_A2, s), 8.4 (H_A8, s), 6.0

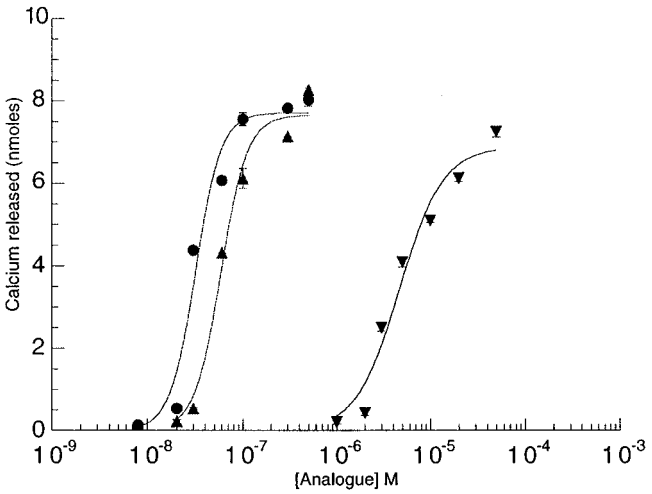

FIGURE 6: Concentration response curves for cADPR, 2′$_A$-deoxy-cADPR, and 3′$_A$- deoxy-cADPR. Assay homogenate (500 μL, 2.5%) was challenged with 5 μL of increasing concentrations of cADPR (●), 2′$_A$-deoxy-cADPR (▲), and 3′$_A$-deoxy-cADPR (▼) to give the final concentrations shown. Fluo-3 fluorescence changes were recorded and correlated to Ca^{2+} release following calibration of assay homogenate with exogenous applications of Ca^{2+}. The values shown relate to means ± SD of triplicate determinations. EC_{50} values were obtained using curve-fitting software (KaleidaGraph) and estimated to be 32 ± 4 nM, 58 ± 5 nM, and 5 ± 1 μM for cADPR, 2′$_A$-deoxy-cADPR, and 3′$_A$-deoxy-cADPR, respectively.

(H1′, d, J = 6 Hz), 5.9 (H_A1′, d, J = 3 Hz), 5.2 (H_A2′, m), 4.7 (H2′), 4.5 (H4′, H5′), 4.4 (H3′), 4.2 (H_A5′, H_A4′), 4.0 (H5′), 3.8 (H_A5′), 2.8 (H_A3′), and 2.1 (H_A3′). ^{31}P NMR (D_2O, 400 M Hz): δ −11.56 and −12.44 (2 P, J_{PP} = 15.13 Hz). MS: *m/z* (+ve ion FAB) 526 $(M + H)^+$ and 627 $(M + TEA)^+$; *m/z* (−ve ion FAB) 524 $(M - H)^-$ and 1049 $(2M - H)^-$. λ_{max}(pH 8.3) 259 nm.

*(D) Synthesis of 3′$_A$-OMe-cADPR (**14**).* 3′$_A$-OMe-NAD^+ (2 × 2.5 μmol) was dissolved in 2 mL (each) of 25 mM HEPES-NaOH buffer, pH 6.8. *Aplysia* cyclase (20 μL, 10 mg of protein/mL concentration) was added to each solution. The mixtures were left to incubate for 20 min, after which the solutions were combined and diluted with 100 mL of MilliQ water and the products were purified by ion-exchange chromatography using a gradient of 0−200 mM TEAB. Residual product 3′$_A$-OMe-NAD^+ eluted between 60 and 70 mM, followed by 3′-OMe-cADPR between 140 and 150 mM TEAB. Pure product was obtained in 40% (2.0 μmol) yield and was quantified by measuring UV absorbance at 254 nm using an extinction coefficient of 14 300 M^{-1} cm^{-1}. HPLC retention times were measured using a 3 × 0.46 cm guard column in combination with a 10 × 0.46 cm Partisil 10 μm SAX column.

R_t (incubation mixture after 20 min): 1.7 (nicotinamide), 2.5 (3′$_A$-OMe-NAD^+), and 4.4 min (3′$_A$-OMe-cADPR). ^{1}H NMR (D_2O, 400 MHz): δ 8.9 (H_A2, s), 8.3 (H_A8, s), 6.0 (H1′, d, J = 3.6 Hz), 5.9 (H_A1′, d, J = 5.5 Hz), 5.3 (H_A2′, dd, J = 5.5 and 5.2 Hz), and 4.7−3.8 (9 H, ribose protons H2′, -3′, -4′, -5′, and -5′ and H_A3′, -4′, -5′, and -5′). ^{31}P NMR (D_2O, 400 MHz): δ −10.55 and −11.55 (2 P). MS: *m/z* (−ve ion FAB) 554 $(M - H)^-$. λ_{max} (pH 8.3) 256 nm.

*(E) Synthesis of 3′$_A$-P-cADPR (**15**).* 3$_A$′- NADP (2 × 3 μmol) was dissolved in 2 mL (each) of 25 mM HEPES-NaOH buffer, pH 6.8. *Aplysia* cyclase (20 μL, 10 mg of protein/mL concentration) was added to each solution. The mixtures were left to incubate for 20 min at room temperature after which the solutions were combined and diluted with 100 mL of MilliQ water. The cyclic product was purified by ion-exchange chromatography using a gradient of 0−500 mM TEAB. Pure 3′$_A$-P-cADPR eluted between 120 and 145 mM TEAB, in 54% yield (3.2 μmol), and was quantified by measuring UV absorbance at 254 nm using an extinction coefficient of 14 300 M^{-1} cm^{-1}. HPLC retention times were measured using a 3 × 0.46 cm guard column in combination with a 10 × 0.46 cm Partisil 10 μm SAX column.

R_t (incubation mixture after 20 min): 2.1 (nicotinamide), 15.9 (3′$_A$-P-NAD^+), and 15.9 min (3′$_A$-P-cADPR). ^{1}H NMR (D_2O, 400 MHz): δ 8.9 (H_A2, s), 8.3 (H_A8, s), 6.0 (H1′, d, J = 3.7 Hz), 5.98 (H_A1′, d, J = 7.3 Hz), 5.3 (H_A2′, dd, J = 5.0 and 7.0 Hz) and 4.6−3.9 (9 H, ribose protons H2′, -3′, -4′, -5′, and -5′ and H_A3′, -4′, -5′, and -5′). ^{31}P NMR (D_2O, 400 MHz): δ +1.0 (1 P, s), −11.0 and −11.75 (2 P, J_{PP} = 22.6 Hz). *m/z* (+ve ion FAB) 723.1 $(M + TEA)^+$ and 622 $(M + H)^+$; *m/z* (−ve ion FAB) 620.1 $(M - H)^-$. λ_{max} (pH 8.3) 256 nm.

*(F) Synthesis of 2′$_A$,3′$_A$-Cyclic- cADPRP (**16**).* 2′$_A$,3′$_A$-cyclic-NADP (2 × 3 μmol) was dissolved in 2 mL (each) of 25 mM HEPES-NaOH buffer, pH 6.8. *Aplysia* cyclase (20 μL, 10 mg of protein/mL concentration) was added to each solution. The mixtures were left to incubate for 20 min, after which solutions were combined and diluted with 100 mL MilliQ water. The products were separated and purified by ion-exchange chromatography using a gradient of 0−400 mM TEAB. 2′$_A$,3′$_A$-Cyclic-NADP eluted between 180 and 190 mM, followed by 2′$_A$,3′$_A$-cyclic-cADPRP between 260 and 290 mM TEAB. Pure product was obtained in 45% (2.7 μmol) yield and was quantified by measuring UV absorbance at 254 nm using an extinction coefficient of 14 300 M^{-1} cm^{-1}. HPLC retention times were measured using 3 × 0.46 cm guard column in combination with a 10 × 0.46 cm Partisil 10 μm SAX column.

R_t (incubation mixture after 20 min): 2.1 (nicotinamide), 8.3 (2′$_A$,3′$_A$-cyclic-cADPRP), and 9.0 min (2′$_A$,3′$_A$-cyclic-NADP). ^{1}H NMR (D_2O, 400 MHz): δ 8.7 (H_A2, s), 8.1 (H_A8, s), 6.2 (H1′, s), 5.9 (H_A1′, d), 5.3 (H_A2′, dd, J = 8.0 and 6.4), 5.5 (H_A3′, dd, J = 4.3 and 5.8), and 4.6−3.9 (8 H, ribose protons H2′, -3′, -4′, -5′, and -5′, and H_A4′, -5′, and -5′). ^{31}P NMR (D_2O, 400 MHz): δ +19.0, (1 P, s), −10.8, and −11.2 [2 P J_{PP} = 13.8 Hz (^{1}H-decoupled)]. *m/z* (+ve ion FAB) 604 $(M + H)^+$; *m/z* (−ve ion FAB) 602.1 $(M - H)^-$. λ_{max} (pH 8.3) 256 nm.

Assessment of Ca^{2+} Releasing Activity of Cyclic Nucleotides. Analogue-induced Ca^{2+} mobilization in sea urchin egg homogenates by synthetic analogues was evaluated fluorometrically. Egg homogenate (2.5%) contained the Ca^{2+}-sensitive probe Fluo-3 (3 μM). The homogenates were prepared from *L. pictus* eggs and diluted as previously described (Willmott et al., 1995).

K_m and V_{max} Determination for Cyclization of NAD^+ and 2′$_A$- and 3′$_A$-Deoxy-NAD^+ by ADP-Ribosyl Cyclase. The kinetics of enzymatic conversion of the above analogues in their cyclic products were investigated using an HPLC method. The conversion was followed by measuring the area under the peak for nicotinamide, a byproduct of ADP-ribosyl cyclase activity. Final substrate concentrations between 25 and 500 μM in 25 mM HEPES buffer, pH 6.8, were used. The total volume in the reaction vessel (an eppendorf) was kept at 500 μL for each reaction. The incubation mixture contained 250 μL of substrate with 225 μL of buffer and 25

μL of enzyme to make a total of 500 μL. A final protein concentration of 5 μg/mL was used in the assay for NAD^+ and 2.5 μg/mL for $2_A'$-deoxy-NAD^+ and $3'_A$-deoxy-NAD^+. Each reaction was analyzed every minute over 6 min. The relative area for nicotinamide was measured and a graph of nicotinamide area vs time was plotted at each concentration of substrate used. The reaction was carried out in duplicate. Initial velocity was calculated from the slope of this curve and an average initial velocity obtained from the two sets of data. K_m and V_{max} were obtained from the Lineweaver-Burk plot by standard computer regression techniques. K_m values obtained were 0.07 mM, 0.74 mM and 0.50 mM and V_{max} values were 22.5, 48.0, 23.7 μmoles/mg total protein/min for NAD^+, $2_A'$-deoxy-NAD^+ and $3_A'$-deoxy-NAD^+ respectively.

RESULTS AND DISCUSSION

We have shown here that a carbodiimide-based method for the synthesis of NAD^+ analogues is reasonably effective. The quality of material prepared after purification can be seen from a typical HPLC trace for **7** in Figure 5A. ^{1}H-NMR spectroscopy of the cyclic products confirmed the disappearance of the nicotinamide protons after enzymatic cyclization. NMR and mass spectrometry also confirmed the integrity of the cyclized product in all cases. This indicates that analogues **7**, **8**, and **13** are substrates for ADP-ribosyl cyclase. K_m values obtained for NAD^+, $2'_A$-deoxy-NAD^+, and $3'_A$-deoxy-NAD^+ (0.07, 0.74, and 0.50 mM, respectively) showed that though the enzyme appears to have a higher affinity for the natural substrate NAD^+, loss of the $2_A'$ or $3_A'$ hydroxyl groups has a significant, but not dramatic, effect on the recognition and substrate activity of NAD^+ by ADP-ribosyl cyclase.

The ^{1}H NMR data of cADPR agree with those previously recorded (Wada et al., 1995). Proton chemical shifts were assigned with the aid of ^{1}H−^{1}H COSY experiments for analogues prepared. For **9**, the H_A1' signal was downfield from H1′ and appeared as a triplet − as it coupled to two H_A2' protons, contrary to the signal for **1** and **10**, where H1′ is downfield from H_A1' and both appeared as singlets. Similarly, a characteristic H_A2' triplet signal at δ 5.2 for cADPR (Walseth & Lee, 1993; Wada et al., 1995) was observed. However, for **9** the two H_A2' protons resonated at a lower frequency, δ 3.2 and 2.4, as a multiplet. H_A2' in **10** appeared as a multiplet at δ 5.2. The two H_A3' protons (**10**) also resonated at a lower frequency − δ 2.8 and 2.1; this is consistent with the loss of the deshielding effect from the neighboring hydroxyl group. The ^{1}H NMR chemical shifts for the protons of analogue **14** resembled those of cADPR except for the *O*-methyl protons that resonated at δ 3.5. ^{1}H NMR spectra for **15** and **16** were similar to those in a preliminary report (Zhang et al., 1996). AB-type signals in the ^{31}P NMR spectra of NAD^+ and cADPR analogues support the existence of the pyrophosphate group. There is a difference of ca. 5 Hz in J_{AB} values between NAD^+ and cADPR analogues. The mass of 524, measured as the negative ion $(M - H)^-$ for **9** and **10** using FAB-MS, is consistent with deletion of an oxygen atom in both cases, compared with cADPR $\{540 - (M - H)^-\}$. *m/z* values of 1049 $(M - H)^-$ were characteristic for both deoxy forms, showing possible formation of larger molecular weight structures. This was, interestingly, not observed in the mass spectrum of cADPR. All three compounds **1**, **9**, and **10**

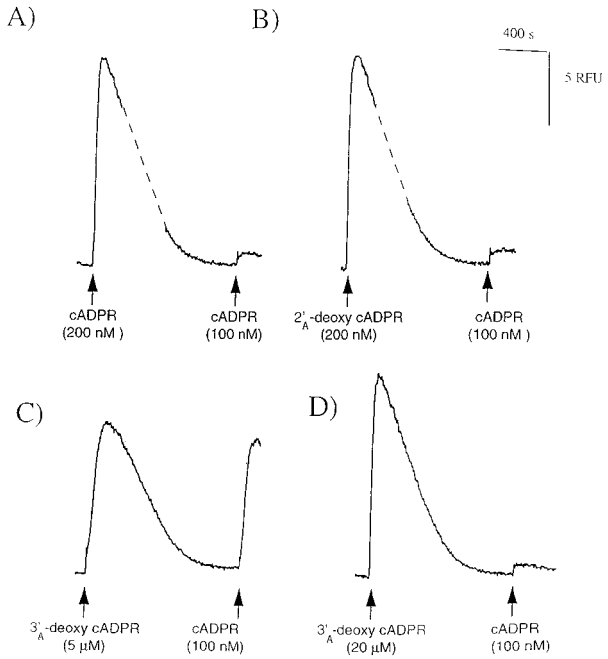

FIGURE 7: Action of analogues on cADPR-sensitive Ca^{2+} release. Fluorescence traces show the Ca^{2+} release from 500 μL of sea urchin egg homogenate (2.5%) (containing the Ca^{2+}-sensitive fluorochrome fluo-3, 3 μM) when challenged with (A) 200 nM cADPR, (B) 200 nM $2'_A$-deoxy-cADPR, (C) 5 μM $3'_A$-deoxy-cADPR, and (D) 20 μM $3'_A$-deoxy-cADPR (final concentrations as shown). $2'_A$-Deoxy-cADPR behaved like authentic cADPR, as 200 nM was sufficient to desensitize to a subsequent addition of cADPR (100 nM). $3'_A$-Deoxy-cADPR must be present in greater amounts to produce the same desensitizing effect (C and D). The traces are from single experiments representative of three similar experiments.

showed a UV absorption maximum at 259 nm; however, there is a decrease in the UV absorption maximum to 256 nm for analogues **14**, **15**, and **16**. An HPLC analysis of the enzymatic conversion of $2'_A$-deoxy-NAD^+ (Figure 5B) illustrates, in addition to the desired product **9**, the production of nicotinamide and $2'_A$-deoxy-ADPR as side products of the cyclization. $2'_A$-Deoxy-ADPR is produced from hydrolytic breakdown of $2'_A$-deoxy-cADPR and was easily removed by ion-exchange chromatography. This is in line with previous reports that suggest *Aplysia* cyclase does exhibit some, albeit low, cADPR hydrolase activity, although the action of other hydrolytic enzymes in the crude extracts cannot be ruled out.

To investigate whether hydroxyl group deletion altered the biological activity of cADPR, we tested the Ca^{2+}-mobilizing ability of both $2'_A$-deoxy-cADPR and $3'_A$-deoxy-cADPR. Like cADPR, the deoxy analogues were Ca^{2+}-mobilizing agonists. However, dose-response curves differed (Figure 6). The concentrations required to produce half-maximal Ca^{2+} release (EC_{50}) were estimated to be 32 ± 4 nM, 58 ± 5 nM, and 5 ± 1 μM for **1**, **9**, and **10**, respectively. A dramatic difference is observed between the dose−response curves for **9** and **10** as shown in Figure 6. $3'_A$-Deoxy-cADPR has a higher EC_{50} value; hence, a higher concentration is required to release the same amount of Ca^{2+} as cADPR. Moreover, 200 nM $2'_A$-deoxy cADPR desensitized cADPR-sensitive Ca^{2+} release channels to subsequent addition of cADPR (100 nM), but 20 μM $3'_A$-deoxy-cADPR was required to produce the same effect (Figure 7). This is in agreement with the 100-fold lower potency exhibited by

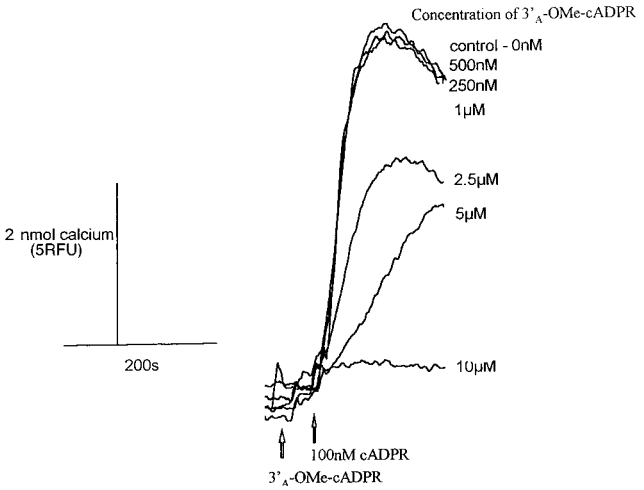

FIGURE 8: Inhibition of cADPR-induced Ca^{2+} release by $3'_A$-OMe-cADPR. Experimental conditions were as described for Figure 6. Fluorescence traces show the effect of pretreating sea urchin egg homogenates (2.5%) with $3'_A$-OMe-cADPR on subsequent cADPR-induced Ca^{2+} release. While $3'_A$-OMe-cADPR is not itself active in releasing Ca^{2+}, it did inhibit Ca^{2+} release by subsequent application of cADPR (100 nM). Concentrations of $3'_A$-OMe-cADPR used ranged from 250 nM to 10 μM as indicated in the figure. This antagonistic activity was concentration-dependent and resembled that previously reported for 8-substituted cADPR analogues, 8-NH_2-cADPR and 8-Br-cADPR (Walseth & Lee, 1993). Approximately half-maximal inhibition (IC_{50}) was produced by one treatment with 5 μM 3′-OMe-cADPR. (Note that the 0 and 500 nM traces are superimposed.)

analogue **10**. Since both analogues are able to prevent cADPR-sensitive Ca^{2+} release, they are likely to be acting to release Ca^{2+} at the cADPR receptor in a similar fashion to cADPR itself. However, it is currently unclear whether this inhibitory effect is due to desensitization of cADPR-sensitive receptors or to store depletion. To test whether this was due to differing rates of analogue breakdown by endogenous cADPR hydrolase, we incubated in control experiments cADPR, $2'_A$-deoxy-cADPR, and $3'_A$-deoxy-cADPR with sea urchin homogenates. HPLC analysis of products over 10 h showed that $3'_A$-deoxy-cADPR was not metabolized faster than cADPR or $2'_A$-cADPR. This suggested that the reduced agonistic activity of **10** is not due to decreased stability or an increased susceptibility to hydrolytic breakdown under the experimental conditions (data not shown). Removal of an oxygen atom from cADPR to give the carbocyclic cyclic aristeromycin diphosphate ribose was recently demonstrated to confer considerable metabolic resistance to this compound relative to cADPR (Bailey et al., 1996a), and deletion of the N7 purine nitrogen results in a compound with partial agonist properties (Bailey et al., 1997). Thus, the present results provide further evidence that small chemical modifications, like hydroxyl group deletion as here, can have a dramatic effect on the biological activity of a cADPR analogue.

Because of the large activity difference observed between **9** and **10** we investigated further the role of the $3'_A$-hydroxyl group. Thus, $3'_A$-OMe-cADPR was synthesized. Unlike the OH moiety, the OMe group is larger and does not possess the ability to donate a proton in a potential H-bond interaction with the receptor, although it can still act as an acceptor. When tested for agonistic activity, $3'_A$-OMe-cADPR was not active in releasing Ca^{2+} in the sea urchin homogenate system (Figure 8). To investigate whether this molecule was interacting with cADPR receptors, we tested its ability to interfere with cADPR-induced Ca^{2+} release. It acted, surprisingly, as an antagonist, in that cADPR-induced Ca^{2+} release was inhibited in a concentration-dependent manner (Figure 8). 3-*O*-Methyladenosine, in contrast, had no such effect up to 100 μM, nor did it act as an agonist for Ca^{2+} release (data not shown). It thus appears that the oxygen atom is somehow important for interaction at the receptor site, as $3'_A$-OMe-cADPR must interact with the receptor in order to inhibit cADPR-induced Ca^{2+} release in a concentration-dependent manner and yet $3'_A$-deoxy-cADPR is a very poor agonist. $3'_A$-Deoxy-cADPR can clearly neither donate nor accept a hydrogen bond at the $3'_A$ position; hence it appears that ability of an analogue to potentially accept a hydrogen bond as in **14** may be important for receptor interaction. It is interesting that mere substitution of the hydrogen atom of the $3'_A$-hydroxyl group of **1** by a methyl group is sufficient to convert the ligand from an agonist to an antagonist.

In contrast, analogues **15** and **16** were not active up to concentrations of 20 μM in mobilizing Ca^{2+} and did not inhibit cADPR-induced Ca^{2+} release in any way, being therefore neither agonists nor antagonists. This is consistent with the results of a previous preliminary study where **15** and **16** were tested for Ca^{2+} release in rat brain microsomes (Zhang et al., 1995) and found to contrast with the agonistic activity of $2'_A$-cADPR phosphate. While no full details of the synthesis or biology have, however, yet been reported by these authors, we provide a much fuller account in this work. $3'_A$-P-cADPR (**15**) is similar in structure to cADPR except for the presence of a phosphate group at the $3'_A$ hydroxyl. $2'_A$,$3'_A$-P-cADPR (**16**) also possesses a cyclic phosphate, substituting both $2'_A$ and $3'_A$ hydroxyls (Figure 1). Analogue **15** also has two extra negative charges and (**16**) has one extra negative charge at physiological pH as compared to cADPR. It is likely that a combination of these factors may result in electrostatic repulsion and steric hinderance of the analogues at the cADPR receptor site, relative to cADPR, thus preventing binding to the receptor. $2'_A$-P-cADPR, an analogue with a phosphate group at the $2'_A$ position, was reported to be active at mobilizing Ca^{2+} from rat brain microsomes (Zhang et al., 1995; Vu et al., 1996) and permeabilized T-lymphocytes (Guse et al., 1997); however, we found it to be inactive in mobilizing Ca^{2+} in sea urchin egg homogenates. This is consistent with a previous report (Aarhus et al., 1995) and may reflect subtle differences in the cADPR-sensitive Ca^{2+} release mechanism for the ryanodine receptor of sea urchin eggs and mammalian tissue. However, we have demonstrated here that replacement of the $2'_A$-OH group with a hydrogen atom has little effect on the activity of cADPR, yet attachment of a phosphate group completely abolishes its activity. This inactivity of $2'_A$-P-cADPR in sea urchin eggs may be due to the above similar factors. It is unclear, however, at present whether the reason lies with the removal of the -OH proton by substitution or rather is the result of charge addition and/or increase in steric volume by addition of a phosphate.

In summary, our results show that $2'_A$-deoxy-NAD^+ and $3'_A$-deoxy-NAD^+ are substrates for ADP-ribosyl cyclase and that the $2'_A$ and $3'_A$ hydroxyl groups appear to play some role, but not an essential one, in the functional recognition of NAD^+ by ADP-ribosyl cyclase. In cADPR, noting the results with $2'_A$-P-cADPR discussed above, the $2'_A$-OH group

appears to be unimportant, and the deoxygenated analogue exhibited very similar Ca^{2+} release properties to cADPR itself. In contrast, the $3'_A$-OH group is necessary for effective Ca^{2+} release activity in sea urchin egg homogenates. Loss of this latter motif resulted in a 100-fold reduction in potency. Substitution of the $3'_A$-hydroxyl group with a methyl group did not prevent receptor interaction but instead converted the molecule from an agonist to an antagonist. This further suggests that the $3'_A$-OH group is important for Ca^{2+} releasing activity in the sea urchin system. In addition, $3'_A$-OMe-cADPR is the first cADPR antagonist that is not modified at the 8-position of the purine ring of cADPR. This surprising property of $3'_A$-OMe-cADPR therefore provides a new lead compound for the design of antagonists of cADPR-induced Ca^{2+} release. Furthermore, the contrasting action of $2'_A$-P-cADPR may offer a new group of molecules for identifying and differentiating tissue- and/or species-specific cADPR binding proteins. Most work to date has concentrated upon structural modification to the purine ring of cADPR (Walseth et al., 1993; Graeff et al., 1996). These results now set the stage for establishing a wider structure−activity profile for cADPR and for developing new pharmacological tools to investigate and modulate the putative signaling pathway mediated by cADPR.

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