Novel Hydrolysis-Resistant Analogues of Cyclic ADP-ribose: Modification of the "Northern" Ribose and Calcium Release Activity†

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ABSTRACT: Three novel analogues modified in the "northern" ribose (ribose linked to N1 of adenine) of the Ca^{2+} mobilizing second messenger cyclic adenosine diphosphoribose, termed 2″-NH_2-cyclic adenosine diphosphoribose, cyclic adenosine diphospho-carbocyclic-ribose, and 8-NH_2-cyclic adenosine diphospho-carbocyclic-ribose, were synthesized (chemoenzymatically and by total synthesis) and spectroscopically characterized, and the pK_a values for the 6-amino/imino transition were determined in two cases. The biological activity of these analogues was determined in permeabilized human Jurkat T-lymphocytes. 2″-NH_2-cyclic adenosine diphosphoribose mediated Ca^{2+} release was slightly more potent than that of the endogenous cyclic adenosine diphosphoribose in terms of the concentration−reponse relationship. Both compounds released Ca^{2+} from the same intracellular Ca^{2+} pool. In addition, the control compound 2″-NH_2-adenosine diphosphoribose was almost without effect. In contrast, only at much higher concentrations ($\geq 50\ \mu$M) did the "northern" carbocyclic analogue, cyclic adenosine diphospho-carbocyclic-ribose, significantly release Ca^{2+} from permeabilized T cells, whereas the previously reported "southern" carbocyclic analogue, cyclic aristeromycin diphosphoribose, was slightly more active than the endogenous cyclic adenosine diphosphoribose. Likewise, 8-NH_2-cyclic adenosine diphospho-carbocyclic-ribose, expected to antagonize Ca^{2+} release as demonstrated previously for 8-NH_2-cyclic adenosine diphosphoribose, did not inhibit cyclic adenosine diphosphoribose mediated Ca^{2+} release. This indicates that the 2″-NH_2-group substitutes well for the 2″-OH-group it replaces; it may be oriented toward the outside of the putative cyclic adenosine diphosphoribose receptor binding domain and/or it can potentially also engage in H bonding interactions with residues of that domain. In sharp contrast to this, replacement of the endocyclic furanose oxygen atom by CH_2 in a carbocyclic system obviously interferes with a crucial element of interaction between cyclic adenosine diphosphoribose and its receptor in T-lymphocytes.

Cyclic adenosine diphosphoribose (cADPR;[1] Figure 1) is a potent calcium-mobilizing, endogenous compound which was discovered by Lee and co-workers (*1, 2*). The structure of this new metabolite of NAD^+ was solved unambiguously by X-ray crystallography in 1994 (*3*). In recent years, the importance of cADPR for the regulation of intracellular Ca^{2+} signaling in a wide variety of cell types covering protozoa, invertebrates and vertebrates, humans, and plants has been demonstrated (reviewed in *4*−*8*). Besides cADPR, D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3; ref *9*] and nicotinic acid adenine dinucleotide phosphate ($NAADP^+$; ref *10*) are also involved in the regulation of intracellular Ca^{2+} signaling. However, the exact roles of each of these messengers in the

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[1] Abbreviations: ADPR, adenosine diphosphoribose; $[Ca^{2+}]_i$, free intracellular Ca^{2+} concentration; cADPR, cyclic adenosine diphosphoribose; cArisDPR, cyclic aristeromycin diphosphoribose; cADPcR, cyclic adenosine diphospho-carbocyclic-ribose; cATPR, cyclic adenosine triphosphoribose; cGDPR, cyclic guanosine diphosphoribose; cGMP, 3′,5′-cyclic guanosine monophosphate; IC_{50}, half-maximal inhibitory concentration; $InsP_3$, D-*myo*-inositol 1,4,5-trisphosphate; IM, ionomycin; ER, endoplasmic reticulum; $NAADP^+$, nicotinic acid adenine dinucleotide phosphate; NADase, NAD^+-glycohydrolase; $NAD(P)^+$, nicotinamide adenine dinucleotide (phosphate); NGD^+, nicotinamide guanine dinucleotide; 2″-NH_2-ADP-ribose, adenosine 5′-diphospho 2″-deoxy-2″-aminoribose; 2″-NH_2-cADPR, ADP-cyclo[N1,-C1″]-2″-deoxy-2″-aminoribose; 2′-P-cADPR, cyclic adenosine diphosphoribose 2′-phosphate; 2″-NH_2-NAD^+, 2″-deoxy-2″-aminoribo-NAD^+ (nicotinamide 2″-deoxy-2″-aminoribose adenine dinucleotide); PLC, phospholipase C; RyR, ryanodine receptor(s); TCR/CD3 complex, T cell receptor/CD3 complex.

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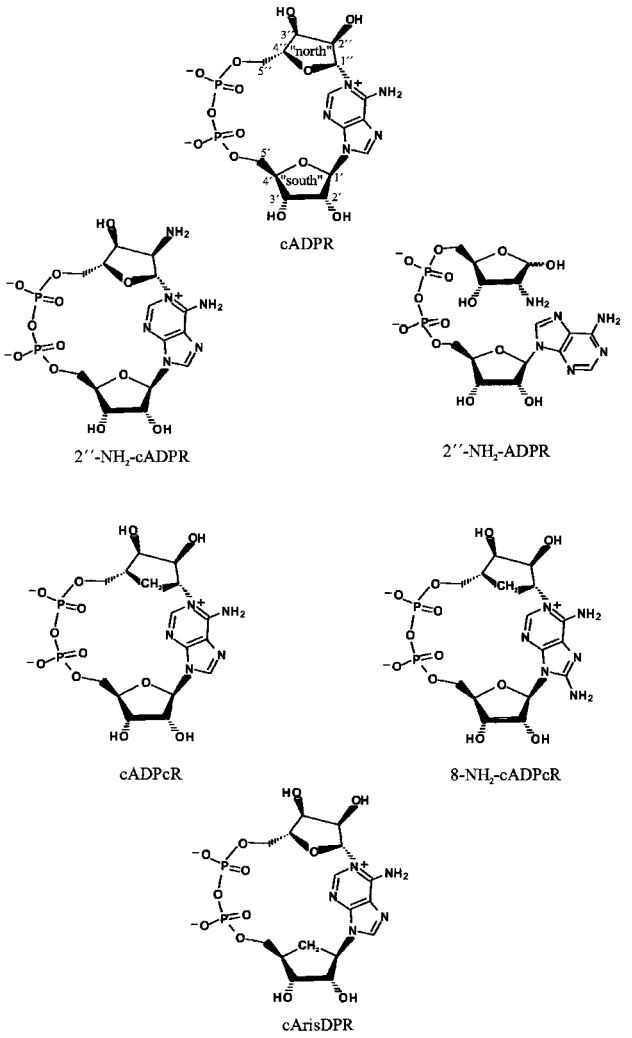

FIGURE 1: Structure of cADPR and analogues used in this study. Note the numbering of the two ribose moieties and the definition of "northern" and "southern" ribose as exemplified for cADPR.

generation of the complex elementary and global pattern of Ca^{2+} signals observed in cells, e.g., spatial events such as Ca^{2+} waves or temporal events such as Ca^{2+} oscillations, have not yet been clearly identified.

A variety of cADPR analogues have been synthesized and tested for their Ca^{2+} mobilizing or antagonistic activity (reviewed in *7*, *8*, *11*). The first and very important analogues were derivatized at the C8-position of the adenine moiety; these compounds, e.g., 8-NH_2- and 8-Br-cADPR, are potent antagonists (*12*). In the meantime, further 8-substituted antagonists, e.g., 8-OCH_3-cADPR, 8-CH_3-cADPR, and 7-deaza-8-Br-cADPR, have been shown to be powerful tools to unravel the role of cADPR in cellular Ca^{2+} signaling (*13*−*15*). Importantly, modifications in the adenine ring also produced agonists, such as 7-deaza-cADPR (*15*), 3-deaza-cADPR (*16*), and 8-aza-9-deaza-cADPR (A. H. Guse and B. V. L. Potter, unpublished results).

Analogues modified only in the "southern" ribose of cADPR (the ribose linked to N9 of adenine; Figure 1) had quite different effects depending on the cell systems. In mammalian cells, e.g., brain microsomes, and permeabilized T cells, 2′-P-cADPR proved to be a Ca^{2+}-mobilizing agonist of similar potency as compared to cADPR (*14*, *17*, *18*), whereas no Ca^{2+} release was seen in lower eukaryotes, e.g., sea urchin eggs (*19*). On the other hand, 2′-deoxy-cADPR was reported to be a potent Ca^{2+}-mobilizing compound in sea urchin eggs (*20*), but was almost without effect in permeabilized T cells (A. H. Guse and B. V. L. Potter, unpublished results). Further derivatives of the "southern" ribose include cyclic aristeromycin diphosphoribose (cArisDPR), a carbocyclic analogue of cADPR with full and potent agonistic activity in sea urchin eggs (*21*), and the 3′-derivatives 3′-phospho-cADPR, 3′-deoxy-cADPR, and 3′-OCH_3-cADPR (*14*, *20*). Another interesting modification was the introduction of a triphospho bridge replacing the natural pyrophosphate of cADPR. The resulting molecule, cyclic adenosine triphosphoribose (cATPR), is a potent Ca^{2+}-mobilizing agonist, at least in bovine brain microsomes (*22*).

In contrast to the "southern" ribose, no cyclic derivatives modified in the other "northern" ribose (the ribose linked to N1 of adenine; Figure 1) have yet been published. Thus, we synthesized three novel cADPR analogues using both a chemoenzymatic approach and total chemical synthesis, one containing an NH_2-group at the 2″-position (2″ indicates a position in the N1-linked ribose and 2′ in the N9-linked ribose to adenine, respectively; Figure 1), termed 2″-NH_2-cADPR (Figure 1), and the others being the carbocyclic analogue cyclic adenosine diphospho carbocyclic ribose (cADPcR) and its 8-amino derivative (Figure 1). The latter two are of particular interest, since they are expected to be nonhydrolyzable by cADPR hydrolase, while being structurally very close mimics of cADPR and 8-NH_2-cADPR, respectively. On the other hand, 2″-NH_2-cADPR is a slowly hydrolyzable analogue of cADPR (*23*), and, if it displays Ca^{2+}-releasing activity, it would be a suitable precursor to synthesize affinity tools for cADPR-binding proteins. These novel analogues were thus characterized spectroscopically, and then their biological activity was determined in Ca^{2+} release experiments in permeabilized human Jurkat T cells.

EXPERIMENTAL PROCEDURES

Materials. NAD^+, ADP-ribose, cyclic ADP-ribose, AMP, nucleotide pyrophosphatase from *Crotalus atrox* venom, CHAPS, saponin, ATP, Chelex resin, and creatine phosphate were obtained from Sigma (either L'Isle d'Abeau Chesnes, France, or Deisenhofen, Germany). cArisDPR was synthesized as described and used as its triethylammonium salt (*21*). Fura2/free acid was from Calbiochem, Bad Soden, Germany. Creatine kinase was supplied by Roche Molecular Biochemicals, Mannheim, Germany. Highly purified water (Seral-Pur) was used for the preparation of all buffers. Glutamax I, newborn calf serum, penicillin, and streptomycin were all from Life Technologies, Eggenstein, Germany.

ADP-ribosyl cyclase was purified, with some modifications, according to published procedures (*24*−*26*) from *Aplysia californica* ovotestis obtained from Dr. Bernard Poulain (CNRS-Neurochimie, Strasbourg, France). The enzyme was stored at −20 °C in 10 mM potassium phosphate buffer, pH 7.4. NAD^+ glycohydrolase from bovine spleen was purified routinely according to our previously published procedure (*27*). 2″-NH_2-NAD^+ was prepared as described (*28*).

Synthesis of 2″-NH_2-cADPR and 2″-NH_2-ADPR. 2″-NH_2-cADPR and 2″-NH_2-ADPR (Figure 1) were obtained from 2″-NH_2-NAD^+ using *A. californica* ADP-ribosyl cyclase and

bovine spleen NAD^+ glycohydrolase, respectively, as described previously (*23*). MS: *m*/*z* (negative ion ES-MS) 539.2 $(M - H)^-$ and 557.2 $(M - H)^-$, respectively, for 2″-NH_2-cADPR and 2″-NH_2-ADPR; *m*/*z* (positive mode) 563.5 $(M + Na)^+$ for 2″-NH_2-cADPR. ^1H NMR (D_2O, 500 MHz Bruker ARX) spectrum of 2″-NH_2-cADPR (ammonium salt) obtained at 293 K: δ 8.99 (H2, s), 8.45 (H8, s), 6.16 (H1′, d, $J = 5.57$ Hz), 6.10 (H1″, d, $J = 5.59$ Hz), 5.50 (H2′, dd, $J = 5.31$ and 5.24 Hz), 4.8−4.1 (H3′, 4′, 5_a′, and 5_b′; and H2″, 3″, 4″, 5_a″, and 5_b″). The proton assignment of the representative downfield region comprising the aromatic and the anomeric protons was obtained by an off-resonance version of 2D ROESY (*30*). A strong NOE was observed between the H8 and the H1′ protons whereas a much lesser signal was found between H2 and H1″. The molar extinction coefficient of 2″-NH_2-cADPR was determined by total phosphate analysis (*31*): at 259 nm (λ_{max}) and pH 7.4, the ϵ value was 13 230 ± 320 M^{-1} cm^{-1} ($n = 4$).

Synthesis and Characterization of cADPcR and 8-NH_2-cADPcR. Briefly, cADPcR and 8-NH_2-cADPcR (Figure 1) were synthesized using an $AgNO_3$-promoted condensation reaction (*32*, *33*) with a phenylthiophosphate-type substrate to form an intramolecular pyrophosphate linkage as a key step. The pyrimidine ring-closure reaction of a sugar-protected imidazole nucleoside, prepared from AICA-riboside, with a known chiral carbocyclic amine gave the desired adenosine derivative. This was further converted into the 5′-phenylthiophosphoryl 5″-phosphate derivative, intramolecular condensation of which with $AgNO_3$ successfully afforded the desired cyclization product. The subsequent deprotection gave cADPcR (*34*). A detailed ^1H NMR analysis of cADPcR suggested that its conformation in aqueous medium is similar to that of cADPR (*34*). 8-NH_2-cADPcR was prepared from the 8-chloro-cADPcR derivative, which was synthesized by a route similar to that for cADPcR, using the corresponding 2-chloro-imidazole nucleoside. Full synthetic details for 8-NH_2-cADPcR will be reported elsewhere.

Cell Culture. Jurkat T-lymphocytes (subclone JMP) were cultured as described previously (*35*) at 37 °C in a humidified atmosphere in the presence of 5% CO_2 in RPMI 1640 medium containing Glutamax I, buffered by HEPES (20 mM, pH 7.4) and supplemented with newborn calf serum (7.5%), penicillin (100 units/mL), and streptomycin (50 μg/mL).

Ca^{2+} Release Assay Using Permeabilized Jurkat T-lymphocytes. Jurkat T cells were permeabilized as described (*36*) except that the saponin concentration (final) was 40 μg/mL and the incubation period with saponin was between 17.5 and 22 min.

Fluorescence measurements using a 900 μL cell suspension (containing approximately 1.2×10^7 permeabilized cells) in the presence of 1 μM fura2/free acid were carried out in a 1 mL quartz fluorescence cuvette at pH 7.2 in an intracellular buffer as described (*36*). Fluorescence was recorded in a Hitachi F2000 instrument with wavelength settings of 340 and 380 nm for excitation (alternating) and 495 nm for emission.

Each experiment was started by addition of creatine kinase (20 units/mL final concentration) and creatine phosphate (20 mM final concentration). Subsequent addition of ATP (1 mM final concentration) completed uptake of Ca^{2+} ions into stores, and the resulting basal Ca^{2+} concentration was usually between 100 and 500 nM, depending on the individual preparation. At the beginning of each series of experiments, the quality of the permeabilized cell preparation was checked by its responsiveness to Ins(1,4,5)P_3 (4 μM final concentration) and cADPR (10 μM final concentration). Chelex resin was added generally to reagent solutions, e.g., cADPR, Ins(1,4,5)P_3, cADPR analogues, to remove contaminations of Ca^{2+}.

RESULTS

Synthesis and Characterization of 2″-NH_2-cADPR. 2″-NH_2-cADPR (Figure 1) was synthesized enzymatically from 2″-NH_2-NAD^+ using highly purified *Aplysia californica* ADP-ribosyl cyclase. Because the cleavage rate of the 2″-deoxy-2′-aminoribose−nicotinamide bond is about 2 orders of magnitude lower than the corresponding bond in NAD^+ (*28*), high amounts of enzyme were needed; under these experimental conditions, the formation of small proportions of 2″-NH_2-ADPR was also observed which could be easily eliminated by a HPLC purification step. The identity of the purified 2″-NH_2-cADPR was confirmed by electrospray ionization mass spectrometry operated both in the positive and in the negative modes; the *m*/*z* values of, respectively, 563.5 $(M + Na)^+$ and 539.2 $(M - H)^-$ are consistent with the expected values. The ^1H NMR data are also in agreement with the structure of the cyclic analogue (Figure 1). Proton chemical shifts of the signature aromatic region and of the anomeric carbons were assigned with the aid of ^1H-^1H ROESY experiments. The H8 proton (δ 8.45) gave a strong NOE with the H1′ proton (δ 6.16). As expected for N1-substitued adenines (*37*), the H2 proton (δ 9.0) was shifted downfield ($\Delta\delta \approx +0.6$ ppm) compared to its chemical shift in the dinucleotide; it also gave a very weak NOE with H1″ (δ 6.10). As found in, e.g., cADPR (*37*), the H2′ was also shifted downfield (δ 5.50). The only noticeable difference of the ^1H NMR spectrum of 2″-NH_2-cADPR compared to those of other cyclic derivatives such as cADPR is the relative order of the anomeric protons; i.e., both appear as doublets with $J = 5.6$ Hz, and the chemical shift of H1″ is slightly upfield, presumably due to the lower shielding effect of NH_2 vs OH.

pK_a Determinations of cADPcR and 2″-NH_2-cADPR. The pK_a values for the 6-amino/imino dissociation of cADPcR and 2″-NH_2-cADPR were determined according to the previously reported method (*38*). The method is based on the UV spectral change between 285 and 300 nm of cADPR and its analogues as shown in Figure 2A for cADPcR. The spectral change is a property of N1-alkyladenine derivatives due to dissociation of a proton from the 6-amino group of the adenine ring. A pK_a of 8.9 was found for cADPcR (Figure 2B), while for 2″-NH_2-cADPR the pH-dependent spectral change at 300 nm showed a pK_a of 9.6 (Figure 2C). Both of these pK_a values are higher than that determined for cADPR (p$K_a = 8.3$; ref *38*).

Biological Activity of 2″-NH_2-cADPR, cADPcR, 8-NH_2-cADPcR, and cArisDPR. Permeabilized Jurkat T-lymphocytes are a well-characterized system to study the biological activity of Ca^{2+}-mobilizing second messengers and their analogues (*7*, *8*). 2″-NH_2-cADPR concentration-dependently released Ca^{2+} from internal stores of permeabilized T cells

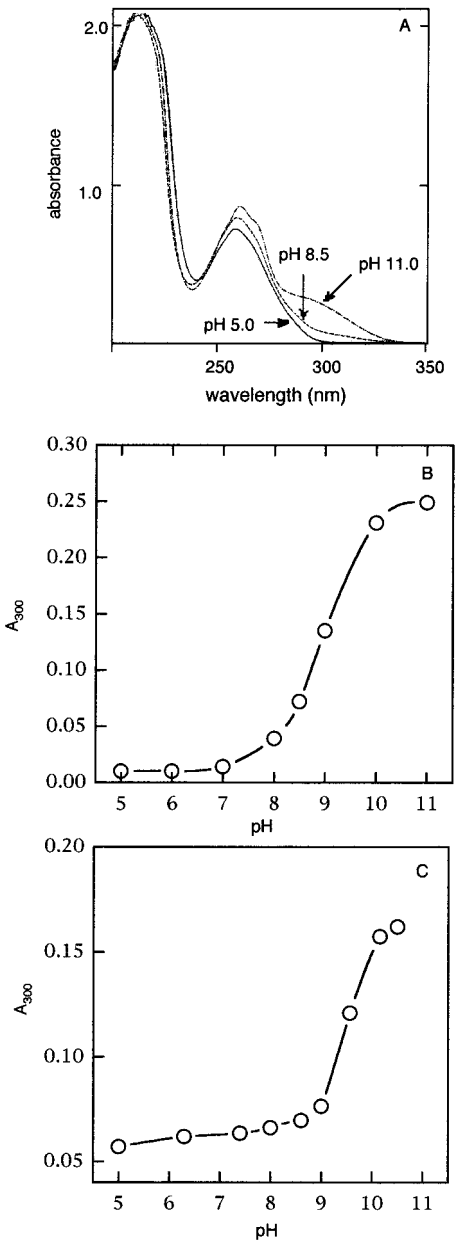

FIGURE 2: Determination of pK_a values of 2″-NH_2-cADPR and cADPcR. pK_a values were determined as described by Kim et al. (*37*) using the increase in the absorbance at 300 nm as an indicator for deprotonation of the NH_2 group at C6 of the adenine ring. (A) UV spectrum of a 0.072 mM solution of cADPcR at different pH values. Plots of absorbance at 300 nm against pH for cADPcR (B) and 2″-NH_2-cADPR (C). Note that different concentrations were used for cADPcR and 2″-NH_2-cADPR.

(Figure 3A): increasing Ca^{2+} release was observed between 1 and 100 μM (Figure 3A). The linear analogue of 2″-NH_2-cADPR, 2″-NH_2-ADPR, up to concentrations of 100 μM was almost inactive regarding Ca^{2+} release (Figure 3B,D); note, however, that the preparation of permeabilized cells normally responded to control cADPR added thereafter (Figure 3B). 2″-NH_2-cADPR-mediated release of Ca^{2+} almost completely abolished a subsequent cADPR-mediated Ca^{2+} release, indicating that both 2″-NH_2-cADPR and cADPR act on the same cADPR-sensitive intracellular Ca^{2+} store (Figure 3C). A quantitative evaluation of the activity of 2″-NH_2-cADPR revealed an EC_{50} of approximately 7 μM as compared to approximately 13 μM for cADPR (Figure 3D).

In contrast to 2″-NH_2-cADPR, the carbocyclic analogue modified in the "northern" ribose, cADPcR, did not significantly release Ca^{2+} when used in concentrations up to 10 μM (Figure 4A), although the permeabilized cell preparations responded well to cADPR and Ins(1,4,5)P_3 (Figure 4B). However, higher concentrations of cADPcR, e.g., 50 and 100 μM, produced a low Ca^{2+} release comparable to 10 and 20 μM cADPR, indicating an approximately 5−10-fold lower biological activity as compared to cADPR (Figure 4A,C). In stark contrast to cADPcR, the carbocyclic derivative of the "northern" ribose, the corresponding derivative of the "southern" ribose, cArisDPR, behaved like a full agonist with an EC_{50} value of approximately 7 μM (Figure 4A,C). 8-NH_2-cADPcR, the carbocyclic analogue of the cADPR antagonist 8-NH_2-cADPR, was also evaluated with regard to its Ca^{2+} release activity using concentrations up to 100 μM; only concentrations of 100 μM produced a significant release of Ca^{2+} (Figure 4C). The EC_{50} values of both cADPcR and 8-NH_2-cADPcR were >100 μM (Figure 4C). Moreover, 50 μM 8-NH_2-cADPcR did not antagonize the Ca^{2+}-mobilizing effect of 10 μM cADPR (Figure 4D), indicating that introduction of a "northern" carbocyclic system (instead of the northern ribose) substantially lowers the affinity of both cADPcR and 8-NH_2-cADPcR toward the cADPR receptor protein.

DISCUSSION

In this report, we describe (i) the synthesis and spectral characterization of three novel cADPR analogues modified in the "northern" ribose, 2″-NH_2-cADPR, cADPcR, and 8-NH_2-cADPcR, and (ii) the biological activity of these compounds and a related carbocylic cADPR analogue, as measured by their Ca^{2+} release activity in permeabilized human Jurkat T cells.

Since the discovery of cADPR, methods have been developed for the synthesis of structurally modified analogues. These have included both chemoenzymatic methods (*12*, *15*−*18*, *20*−*22*) and methods for total synthesis (*32*−*34*). The former have so far proven very successful, but rely totally on the acceptance of structurally modified NAD^+ analogues by *A. californica* ADP-ribosyl cyclase; few exceptions have been found so far, although this may limit the generation of potential diversity. Moreover, the likely much more difficult cyclization of analogues modified in the "northern" ribose has not yet been attempted; one reason for this has been the a priori expectation that modifications close to the site of enzyme-mediated N1-ribosylation would severely affect this process. However, we have shown previously that replacement of the 2″-OH of the "northern" ribose of NAD^+ by susbtituents with different electron acceptor/donor properties yielded analogues that were still substrates of CD38/NAD^+ glycohydrolases (*28*); the ability of these enzymes to catalyze the cleavage of the nicotinamide-modified ribose bonds was, however, highly dependent on the capacity of these substituents to stabilize the intermediary oxocarbenium ion (*28*). Such an effect could also be expected, in principle, for reactions catalyzed by *A. californica* ADP-ribosyl cyclase which shares with the CD38/NAD^+ glycohydrolases a common reaction scheme (*39*). Among the different options available, we selected the 2″-NH_2 substituent, because the analogue 2″-NH_2-NAD^+ could still be converted into 2″-NH_2-cADPR with the *A. californica*

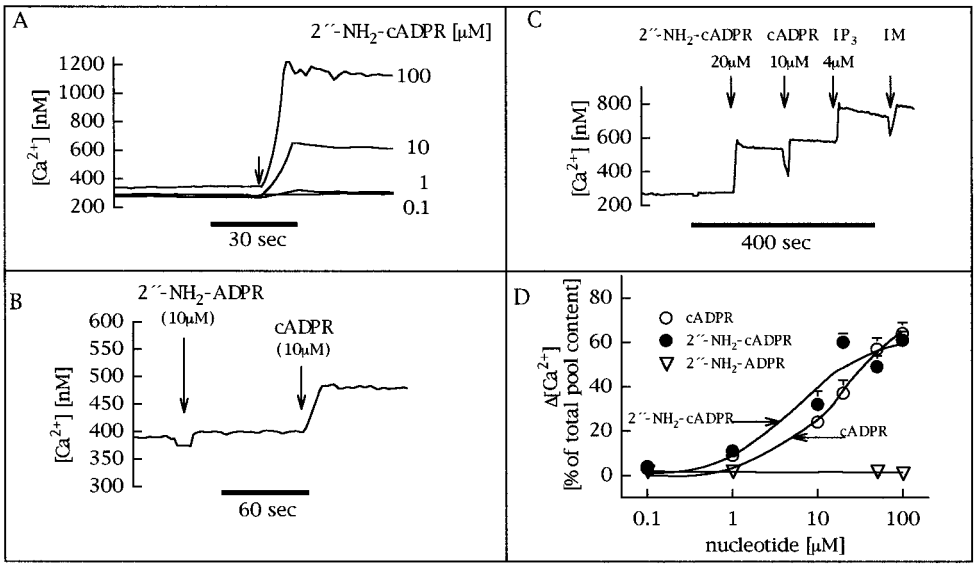

FIGURE 3: Ca^{2+} release by 2′′-NH_2-cADPR from permeabilized Jurkat T cells. Jurkat T cells were permeabilized, and [Ca^{2+}] was determined fluorometrically in the presence of Fura2 as described under Experimental Procedures. (A) Representative tracings of concentration-dependent release of Ca^{2+} by 2′′-NH_2-cADPR. (B) Lack of effect of the linear analogue 2′′-NH_2-ADPR. (C) Inhibition of cADPR-mediated, but not of Ins(1,4,5)P_3-mediated, Ca^{2+} release after initial addition of 2′′-NH_2-cADPR. (D) Concentration−response curves for cADPR, 2′′-NH_2-cADPR, and 2′′-NH_2-ADPR: data are differences (Δ) between the basal Ca^{2+} concentration in the particular experiment and the increase obtained with the concentration of nucleotide indicated expressed as mean ± SEM in % of total Ca^{2+} pool content (n = 3−15 for 2′′-NH_2-cADPR, n = 3−37 for cADPR, n = 2−5 for 2′′-NH_2-ADPR).

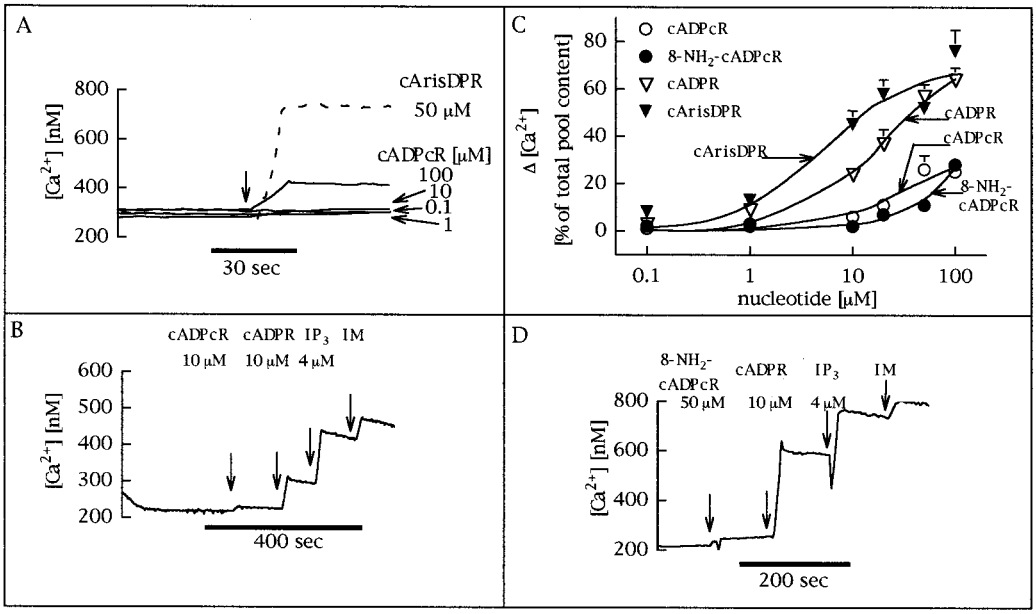

FIGURE 4: Ca^{2+} release by cADPcR, 8-NH_2-cADPcR, and cArisDPR from permeabilized Jurkat T cells. Jurkat T cells were permeabilized, and [Ca^{2+}] was determined fluorometrically in the presence of Fura2 as described under Experimental Procedures. (A) Representative tracings of concentration-dependent release of Ca^{2+} by cADPcR and cArisDPR. (B) Lack of effect of 10 μM cADPcR, whereas 10 μM cADPR and 4 μM Ins(1,4,5)P_3 produced significant responses. (C) Concentration−response curves for cADPR, cADPcR, 8-NH_2-cADPcR, and cArisDPR: data are differences (Δ) between the basal Ca^{2+} concentration in the particular experiment and the increase obtained with the concentration of nucleotide indicated expressed as mean ± SEM in % of total Ca^{2+} pool content (n = 2−5 for cArisDPR, n = 2−6 for cADPcR, n = 3−37 for cADPR, n = 1−4 for 8-NH_2-cADPcR). (D) Lack of antagonistic activity of 50 μM 8-NH_2-cADPcR toward Ca^{2+} release by 10 μM cADPR.

cyclase at reasonable rates. This successful transformation also confirms that the *A. californica* ADP-ribosyl cyclase, perhaps surprisingly, has no stringent structural requirements for its substrates in relation to the nicotinamide-bearing ribose and should thus be useful to produce new analogues of cADPR modified in the "northern" part.

As an alternative to the chemoenzymatic approach, the use of total synthesis to generate cADPR analogues offers a very wide choice of potential structural modifications, but it is likely that complex routes will have to be designed for individual analogues using difficult chemistry that will most likely not be compatible with rapid acquisition of structure−activity relationship information. The two other compounds reported here are the first analogues modified in the "northern" ribose of cADPR to be prepared in this fashion.

Although cADPR has been shown to act as an endogenous Ca^{2+}-mobilizing messenger in three different kingdoms of living organism, e.g., plants, protozoa, and animal cells, the

receptor for cADPR has not yet been identified. According to inhibition studies, the Ca^{2+} release channel responding to cADPR appears to be the ryanodine receptor (RyR; refs *13, 40*). However, whether the RyR directly binds cADPR or if additional cADPR-binding proteins are involved is still a matter of debate. Evidence for the latter was obtained by photoaffinity labeling of sea urchin egg homogenates using [^{32}P]-8-N_3-cADPR. Such experiments resulted in the specific detection of protein bands of 100 and 140 kDa (*41*). In contrast, in pancreatic acinar cells, the RyR-associated protein FKBP12.6 was shown to act as a cADPR-binding protein (*42*). Despite these results, the 3D structure of the binding pocket for cADPR at its binding protein is completely unresolved at the moment.

Since under these conditions rational design of compounds fitting into the putative cADPR-binding domain is impossible, various cADPR analogues have been synthesized, and their biological activity in different cell systems has been determined (see the introduction). Interestingly, compounds structurally modified in the "southern" ribose had different effects in lower vs higher eukaryotes, e.g., sea urchin egg vs human T cells or rat or bovine brain microsomes. The naturally occurring 2′-phospho-cADPR (*43*) released Ca^{2+} from bovine and rat brain microsomes and from permeabilized T cells (*14, 17, 18*), whereas it was without effect in sea urchin homogenates (*19*). On the other hand, the 2′-deoxy-cADPR was a potent agonist in the sea urchin system (*20*), but was only a very weak agonist in permeabilized T cells (A. H. Guse and B. V. L. Potter, unpublished results). These data suggest the presence of different binding proteins for cADPR in lower vs higher eukaryotes, or different binding proteins in different tissues, e.g., oocytes vs neuronal or lymphatic tissue.

In the present study, three novel analogues of cADPR with modifications in the northern ribose and purine ring were analyzed for their biological activity together with one analogue with modifications in the southern ribose. Interestingly, changing the 2″-OH to a 2″-NH_2 group not only retained biological activity, but also made the molecule a little more active as compared to cADPR. This may possibly be a reflection of the hydrolysis resistance of the analogue to conversion to 2″-NH_2-ADPR (but also see later arguments relating to the N6 amino group). Alternatively, this may indicate that the 2″-substituents of the "northern" ribose are either close to the border or outside of the cADPR-binding pocket of the putative cADPR receptor, or, if inside or very close to the binding pocket, that the 2″-NH_2 group can make new hydrogen bonds with neighboring amino acid residues. This latter possibility should take into account the low pK_a value of the 2″-NH_2 group, which in 2″-NH_2-NAD^+ is about 4.6 (Handlon, A. L., and Oppenheimer, N. J., unpublished data). Thus, structurally this can be rationalized by the 2″-NH_2 group making similar potential H-bond contacts with the receptor as the 2″-OH of cADPR. This view is supported by the fact that 2″-NH_2-cADPR released Ca^{2+} from the same pool as compared to cADPR; this clearly suggests that the same binding protein in T cells should be involved.

The analogue cADPcR was, however, much weaker in its biological activity as compared to cADPR. This is a surprising result, since the structural change is much less dramatic than the one in 2″-NH_2-cADPR. However, if the assumption that the 2″-NH_2 group is close to the border or outside the binding pocket is correct, then the "northern" carbocyclic ribose surrogate may still be within the pocket; the orientation of the methylene group of the carbocyclic ring, at least relative to the ribose oxygen in the crystal structure of cADPR (*3*), is almost in the opposite direction to that of the 2″-substituent. If, however, the original endocyclic ribose oxygen of the northern ribose of cADPR itself, by interacting with polar or charged amino acid residues, is important to keep the molecule in the right position within the binding pocket, the methylene of the "northern" carbocyclic analogue, due to its very different physical properties, may lower the affinity considerably. In contrast to cADPcR, the carbocyclic analogue modified in the "southern" ribose, cArisDPR, was a potent full agonist, both in the sea urchin egg system, as earlier determined (*21*), and in permeabilized T cells (this study), indicating that replacement of the endocyclic oxygen in this location is not per se critical for biological activity. Additionally, this molecule showed some evidence of enhanced activity relative to cADPR, consistent with its previously determined slow hydrolysis by ADP-ribosyl cyclase in sea urchin homogenate (*21*). In contrast to our results in human Jurkat T cells, cADPcR was highly potent when microinjected into sea urchin eggs. Indeed, it seemed to be significantly more potent than cADPR itself (*34*), and this was rationalized in terms of its hydrolysis resistance. This result adds further to the conclusion that cADPR-binding proteins must be different between lower and higher eukaryotes, since cADPcR, together with the analogues modified in the 2′-position of the "southern" ribose, e.g., 2′-P-cADPR and 2′-deoxy-cADPR (see the introduction and Discussion above), behaved completely different in the different cellular systems. Note, however, in this regard the consistency of behavior of cArisDPR in sea urchin homogenate and T-lymphocyctes.

The concentration−response relationship obtained for cADPR revealed a somewhat higher EC_{50} value than previously observed in permeabilized T cells (*35*). However, this is likely explained by small differences in the presence and concentration of cytosolic factors, such as calmodulin, which are known as important modulators of cADPR-mediated Ca^{2+} release (*44, 45*).

cADPR can exist in two structural forms at physiological pH, an N6-imino or amino form. It is currently not known which of these represents the active form at the cADPR-receptor, or indeed whether both forms may be active. It has been shown by Jacobson and co-workers that the amino group at the C6-position of the adenine ring of cADPR will be deprotonated under basic conditions with a pK_a of 8.3 (*38*). This leads to the loss of the formal positive charge at N1 of the adenine. Thus, at the pH used for Ca^{2+} release experiments (pH 7.2), the amino:imino form ratio of cADPR is about 12.5:1. This proportion is shifted still further toward the amino form in cADPcR (pK_a 8.9) to 50:1, and even more in 2″-NH_2-cADPR (pK_a 9.6) to 250:1. The increase of the pK_a in cADPcR over that for cADPR is caused by the electron-donating effect of the carbocyclic system as compared to the furanose of cADPR. The larger increase of the pK_a value for 2″-NH_2-cADPR is more difficult to explain; perhaps the effect may be due to the spatial proximity of the 2″-amino and the C6-amino/imino group and the formation of a more extensive hydrogen bond network than in cADPR; this idea is supported by the crystal structure of

cADPR (*3*). It may well be that such spatial juxtaposition stabilizes the amino group at C6, e.g., by stabilizing the charge at N1, thereby shifting the amino-to-imino transition to higher pH values.

Before the biological activity of our analogues was tested experimentally, we hypothesized that the relative proportion of the C6 imino/amino forms might be crucial for the Ca^{2+} release activity of cADPR (and its analogues), e.g., the more imino form, the more Ca^{2+} release activity or vice versa. Other things being equal, if the former assumption was correct, one would expect 2″-NH_2-cADPR to show weaker activity as compared to cADPcR. However, in our experiments this was not the case since 2″-NH_2-cADPR was even more potent than cADPR. Finally, the pK_a determination exclusively of the C6 substituent is based on the assumption that the 285−320 nm shoulder in the UV spectra is only due to changes in the region of N1 of the adenine ring (*38*). While still likely, in the analogues it may, however, be possible that due to the structural modifications there might be additional effects on the spectrum not directly related to the ionization state of the N1 atom. While our results do not permit an unambiguous conclusion to be drawn on the relative activity of the imino/amino forms, our working hypothesis is that the amino form represents the primary active form of cADPR. The enhancement of the population of this form in cADPcR by virtue of the higher pK_a did not result in an activity increase (at least in the T-lymphocyte setting) since this is negated by the loss of a key receptor interaction to the endocyclic furanose oxygen of the northern ribose. However, in 2″-NH_2-cADPR, where the species population of the amino form is the highest, this correlates with the high biological activity of this analogue, and perhaps indeed (if the imino form is not active) with its higher potency in relation to cADPR itself. This may of course be linked also with any effects derived from the stability against hydrolysis mentioned earlier. It is of interest to note that the crystal structure determination (*3*) shows that the N^6-position of the adenine ring is in the imino form. However, the crystal structure is of the crystallized free acid, and this will not be directly relevant to physiological conditions.

Another important characteristic of both analogues is their stability toward hydrolysis. 2″-NH_2-cADPR displays an at least 100-fold slower hydrolysis as compared to cADPR (*23*). cADPcR was stable to enzymic hydrolysis by the hydrolase in rat brain microsomes and by pure extracellular domain of CD38 (*34*). Also, it was chemically stable to acidic and neutral conditions, unlike cADPR (*34*). These characteristics make both compounds rather useful in a biological context, where cell surface enzymes such as CD38 and CD157 (BST-1) and intracellular forms of CD38 (*46*, *47*) rapidly degrade cADPR.

In summary, we demonstrate the synthesis and spectral characterization of three novel cADPR analogues with modifications in the "northern" ribose. For the first time, we show that one such compound, 2″-NH_2-cADPR, and the carbocyclic analogue, cArisDPR, possess even better biological activity than cADPR in a human cell system, in which the importance of the cADPR/Ca^{2+} signaling pathway has been clearly established (*13*). A fully active cADPR analogue with the 2″-amino group also appears to provide an optimal basis to design affinity tools for purification of proteins involved in the cADPR/Ca^{2+} signaling pathway. The poor ability of cADPcR to release Ca^{2+} in permeabilized T cells, on the other hand, provides hints on the 3D structure of the binding pocket for cADPR in that cellular system, as well as providing new structure−activity parameters. Additionally, the fact that potent activity is retained in an analogue modified in the "northern" ribose and synthesized straightforwardly from a modified NAD^+ derivative using ADP-ribosyl cyclase demonstrates that the much simpler route of chemoenzymatic synthesis vs total chemical synthesis may offer a wider versatility than was previously supposed.

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