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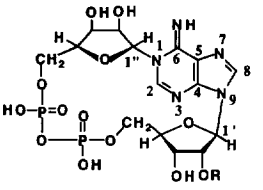
SYNTHESIS AND CHARACTERIZATION OF CYCLIC ATP-RIBOSE: A POTENT MEDIATOR OF CALCIUM RELEASE

Fang-Jie Zhang, Shinji Yamada, Qu-Ming Gu, and Charles J. Sih*
School of Pharmacy, University of Wisconsin-Madison,
425 N. Charter Street, Madison, WI 53706-1515

ABSTRACT: Cyclic ATP-ribose, synthesized by a combination of chemical and enzymatic methods, was twenty times more potent than cADPR in inducing Ca^{2+} release from rat brain microsomes. It induced Ca^{2+} release from the same Ca^{2+} stores as that of cADPR, but different from those of IP_3 and cyclic ADP-ribose-phosphate.

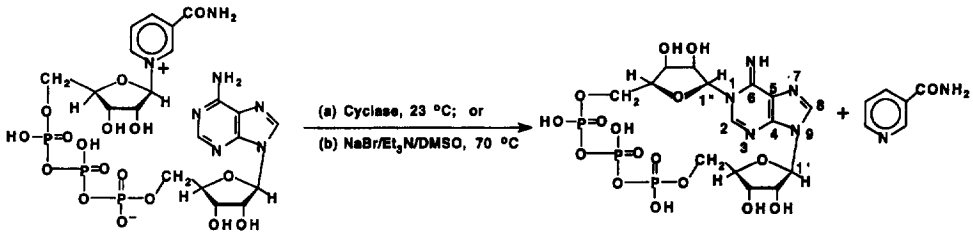
Numerous cellular functions ranging from hormone secretion to neurotransmitter release are regulated by the intracellular cytoplasmic Ca^{2+} concentrations.[1] The intracellular Ca^{2+} stores of cells utilize one or two of the intracellular Ca^{2+} channels to release Ca^{2+} upon cell stimulation. The inositol 1,4,5-triphosphate (IP_3) receptor, which is activated by the second messenger IP_3, mediates calcium release mainly from the endoplasmic reticulum of nonexcitable cells.[2] The calcium release channel of skeletal and cardiac cells is the ryanodine receptor,[3] which may be activated by Ca^{2+} and ryanodine as well as cyclic ADP-ribose (cADPR), **1**, a putative physiological mediator.[4]



1 cADPR R = H

2 cADPRP R = PO_3

To date, only a few structural analogs of **1** have been synthesized and all of them were shown to be less effective than **1** in inducing Ca^{2+} release in various biological systems.[5] The only exception was cyclic ADP-ribose-phosphate (cADPRP) **2**, which was reported to be twice as potent as **1** in mobilizing Ca^{2+} in the rat brain microsomal system.[6] Our continued interest in defining the relationships between structural alterations and biological activities and the preparation of more stable cyclic analogs of **1** for use as affinity probes led us to the synthesis of cyclic ATP-ribose (cATPR) **3**. This homolog was found to be considerably more potent than **1** in inducing Ca^{2+} release and more resistant to the hydrolytic actions of NAD glycohydrolases. The synthesis, characterization, and biological activities of this novel cyclic nucleotide are described herein.



4 5'-TPN **3** cATPR

The synthesis of **3** began with the preparation of the requisite acyclic precursor, 5'-triphosphopyridine nucleotide (5'-TPN), **4**, which was obtained in 42% yield by the condensation of ADP with β-nicotinamide monophosphate (β-NMN) according to a published method.[7] Our initial attempts to cyclize **4** using our non-enzymatic method[8] resulted in the formation of **3** in only 10% yield. However, it was gratifying to find that the ADP-ribosylcyclase from *Aplysia californica* catalyzed the cyclization of **4** to give **3** in high yield. In a representative experiment, 5'-TPN (6.0 mg, 8.1 μmol) was incubated with the cyclase (2 units, Sigma) in 6 mL of 0.1 M $NaHCO_3$ buffer, pH 8.3, for 3 h at 24 °C. The mixture was filtered and the filtrate was concentrated and then chromatographed on a SynChropak AX-100 column (250 × 7.8 mm i.d., 5 μm). The column first was equilibrated with 0.1 M KH_2PO_4 (pH 4.0) (solvent A) and then eluted using a linear gradient consisting of 0.1 M KH_2PO_4 and 1.5 M NaCl (pH 4.0) (solvent B) to 80% for 24 min at a flow rate of 2 mL/min. This was followed by isocratic elution using solvent B until 28 min at a flow rate of 2 mL/min. Fractions containing **3** (retention time 19.6 min) (Figure 1) were combined and lyophilized. The inorganic salts were removed using

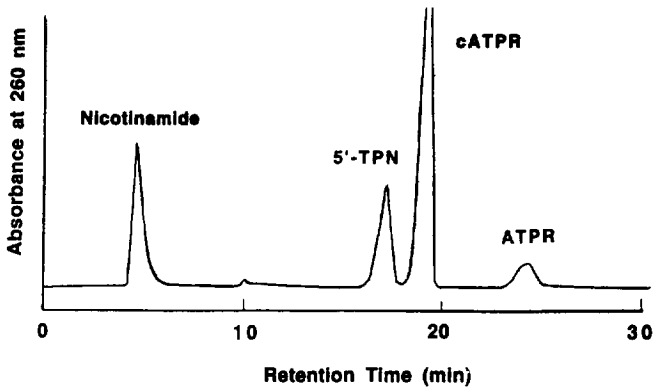


Figure 1. HPLC profile of the reaction of 5'-TPN with ADP-ribosylcyclase after 2 h. The conditions of the reaction and HPLC analysis were carried out as described in the text.

a Waters Nova-Pak C_{18} column (100 × 8 mm i.d., 4 μm), which was eluted isocratically with 3 mM TFA at a flow rate of 0.9 mL/min. Pure **3** was obtained as a white solid in 80% yield. ^1H NMR (D_2O, 300 MHz, pH

3.0) δ 4.10-4.80 (9H, m), 5.33 (1H, t, J = 4.5 Hz, H2′), 6.10 (1H, d, J = 5.4 Hz, H1′), 6.20 (1H, d, J = 3.9 Hz, H1″), 8.44 (1H, s, H8), 9.09 (1H, s, H2). ^{13}C NMR (D_2O, 300 MHz, pH 3.0) δ 151.3 (C6), 148.1 (C4), 147.0 (C8), 143.7 (C2), 121.2 (C5), 94.7 (C1″), 91.6 (C1′), 88.2, 85.8, 77.5, 73.7, 72.5, 71.8, 66.2, 66.0. UV λ_{max} (pH 6.0), 257 (ε 13,000) nm. FAB MS *m/e* 620.0199 ([M-H]$^-$), $C_{15}H_{21}N_5O_{16}P_3$ requires 620.0194.

By analogy to the transformation of β-NAD to cADPR, we assumed that a cyclic nucleotide was similarly generated via the formation of a new glycosyl linkage. This supposition is supported by its ^{1}H NMR spectrum showing the presence of singlets at δ 8.44 and 9.09, corresponding to the H8 and H2 protons of the purine ring, and the FAB high resolution mass spectrum exhibited a molecular ion ([M-H]$^-$) at *m/e* 620.0199. The proton-decoupled ^{13}C spectrum of cATPR exhibited signals for C6 (δ 151.3), C5 (δ 121.2), C4 (δ 148.1), C2 and C8 (δ 143.7 and/or 147.0), and signals for ribosyl carbons in the region of δ 60-100. C2 and C8 signals were more intense than those of C4, C5, and C6 due to the NOEs arising from attached protons. C5 lies upfield of C4 and C6 because it has only one nitrogen substituent, whereas the latter have two. The above assignments are also confirmed by the HMBC spectrum (without suppression of one-bond couplings) as shown in Table 1.

Table 1. ^{1}H-^{13}C connectivities for **3** established by HMBC spectrum

	^{13}C (δ)	H2	H8	H1′	H1″
C2	143.7	HC			HCNC
C4	148.1	HCNC	HCNC	HCNC	
C5	121.2		HCNC		
C6	151.3	HCNC			
C8	147.0		HC	HCNC	
C1′	91.6				
C1″	94.7	HCNC			

Both C2 and C8 show one-bond couplings with the protons attached to them. The distinction of C2 and C8 was made on the basis of the coupling of H8 to C5, and H2 to C6. The long-range couplings also allowed the assignment of the signal for C4 since it is the only carbon with detectable coupling to both H2 and H8. With the purine ring protons and carbons assigned, the three-bond couplings between H1′ and C8 and C4, and between H1″ and C2 revealed that the newly formed glycosyl linkage is attached onto the N-1 position of the purine ring.

Stability of cATPR. The stabilities of cATPR and cADPR were examined at different pH values in different buffers. The results (Figure 2) showed that cATPR was more stable than cADPR, which may be rationalized by the relief of ring strain by the larger ring size. In neutral buffers, both cATPR and cADPR were stable at 24 °C, but hydrolysis became rapid as the temperature was raised.

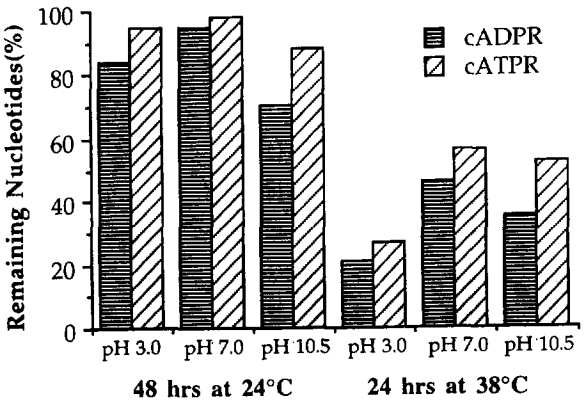


Figure 2. cADPR or cATPR (0.5 mM) was incubated in the respective buffers. Samples were analyzed by anion-exchange HPLC as described in the text. 10 mM acetate buffer (pH 3.0), 10 mM phosphate buffer (pH 7.0), 10 mM glycine-NaOH buffer (pH 10.5).

Since NAD-glycohydrolases (NADases) are widely distributed among mammalian tissues, the relative stabilities of cATPR and cADPR towards the hydrolytic action of this enzyme were compared. As shown in Figure 3, cATPR was more stable than cADPR when incubated with pig brain NADase at 37 °C. After 40 min, 80% of cADPR was hydrolyzed whereas only 30% of cATPR was hydrolyzed under these same conditions. However, both cADPR and cATPR were resistant to the action of nucleotide pyrophosphatase at 24 °C.[9]

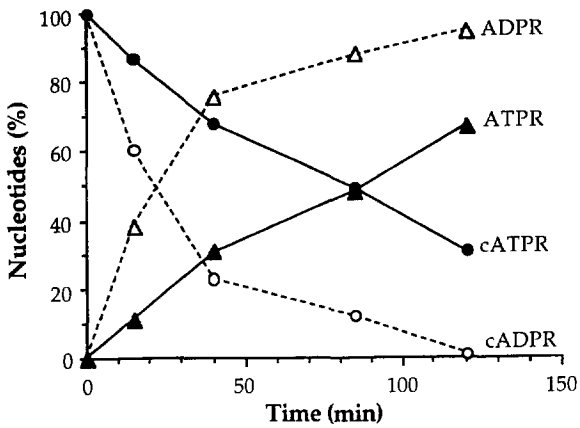


Figure 3. cADPR or cATPR (1 mM) was incubated with pig brain NADase (2 mg/mL, 0.02 U/mg protein) at 37 °C. Samples were analyzed by anion-exchange HPLC as described in the text.

Calcium release. Ca^{2+} release was measured in the rat brain microsomal system as described by White et al.[10] As shown in Figure 4, cATPR was very effective in inducing Ca^{2+} release from rat brain microsomes.

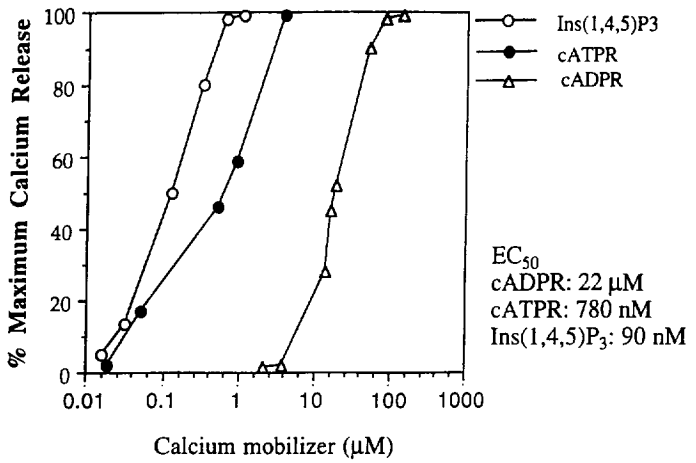


Figure 4. Dose response curve of Ca^{2+} release from rat brain microsomes.

The half-maximal effective concentration (ED_{50}) of cATPR was about 0.8 μM, which is twenty times more potent than cADPR. To determine whether cATPR competes with cADPR for the same binding site, we conducted the following experiments. Addition of cATPR elicited a rapid Ca^{2+} release and after the Ca^{2+} was resequestered, further addition of cATPR or cADPR did not induce more Ca^{2+} release. Likewise, when microsomes were first desensitized by cADPR, further addition of cATPR also did not induce Ca^{2+} release. However, subsequent addition of inositol (IP_3) induced a significant quantity of Ca^{2+} release. These results indicated that cATPR and cADPR modulated the Ca^{2+} release via the same mechanism, but different from that of IP_3 or cADPRP.[6]

In conclusion, we have synthesized a new Ca^{2+} release mediator, cATPR. This analog is more potent in inducing Ca^{2+} release in rat brain microsomes and more stable than cADPR to hydrolysis by NAD-glycohydrolases. More interestingly, cATPR and cADPR act on the same receptor, which suggests that it should be possible to isolate and characterize cADPR-binding proteins using the more effective and stable cATPR as an affinity probe. This work is ongoing and the results will be reported in due course.

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