British Journal of Pharmacology (2006) 149, 337–344

0007–1188/06 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Cellular effects and metabolic stability of *N*1-cyclic inosine diphosphoribose and its derivatives

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Background and purpose: Recently, a number of mimics of the second messenger cyclic ADP-ribose (cADPR) with replacement of adenosine by inosine were introduced. In addition, various alterations in the molecule ranging from substitutions at C8 of the base up to full replacement of the ribose moieties still retained biological activity. However, nothing is known about the metabolic stability and cellular effects of these novel analogues.
Experimental approach: cADPR and the inosine-based analogues were incubated with CD38, ADP-ribosyl cyclase and NAD-glycohydrolase and metabolism was analysed by RP-HPLC. Furthermore, the effect of the analogues on cytokine expression and proliferation was investigated in primary T-lymphocytes and T-lymphoma cells.
Key results: Incubation of cADPR with CD38 resulted in degradation to adenosine diphosphoribose. ADP-ribosyl cyclase weakly catabolised cADPR whereas NAD-glycohydrolase showed no such activity. In contrast, *N*1-cyclic inosine 5′-diphosphoribose *(N*1-cIDPR) was not hydrolyzed by CD38. Three additional *N*1-cIDPR analogues showed a similar stability. Proliferation of Jurkat T-lymphoma cells was inhibited by *N*1-cIDPR, *N*1-[(phosphoryl-*O*-ethoxy)-methyl]-*N9*-[(phosphoryl-*O*-ethoxy)-methyl]-hypoxanthine-cyclic pyrophosphate (*N*1-cIDP-DE) and *N*1-ethoxymethyl-cIDPR (*N*1-cIDPRE). In contrast, in primary T cells neither proliferation nor cytokine expression was affected by these compounds.
Conclusions and Implications: The metabolic stability of *N*1-cIDPR and its analogues provides an advantage for the development of novel pharmaceutical compounds interfering with cADPR mediated Ca^{2+} signalling pathways. The differential effects of *N*1-cIDPR and *N*1-cIDPRE on proliferation and cytokine expression in primary T cells versus T-lymphoma cells may constitute a starting point for novel anti-tumor drugs.
British Journal of Pharmacology (2006) **149,** 337–344. doi:10.1038/sj.bjp.0706869; published online 11 September 2006

Keywords: cyclic ADP-ribose; ADP-ribosyl cyclase; CD38; T-lymphoma cell; primary T cell; stable analogues; calcium signalling; proliferation; cytokine expression; signal transduction

Abbreviations: 8-Br-*N*1-cIDPR, 8-bromo-cyclic inosine 5′-diphosphoribose; ADPR, adenosine 5′-diphosphoribose; ADPRC, ADP-ribosyl cyclase; cADPR, cyclic adenosine 5′-diphosphoribose; cGDPR, cyclic guanosine 5′-diphosphoribose; EC_{50}, effector concentration for half-maximum response; GFP, green fluorescent protein; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]; HPLC, high-performance liquid chromatography; IDPR, inosine 5′-diphosphoribose; MBP, myelin basic protein; *N*1-cIDP-DE, *N*1-[(phosphoryl-*O*-ethoxy)-methyl]-*N9*-[(phosphoryl-*O*-ethoxy)-methyl]-hypoxanthine-cyclic pyrophosphate; *N*1-cIDPR, *N*1-cyclic inosine 5′-diphosphoribose; *N*1-cIDPRE, *N*1-ethoxymethyl-cIDPR; NAD, nicotinamide adenine dinucleotide; NADase, NAD glycohydrolase; NGD, nicotinamide guanine dinucleotide; RT, room temperature

Introduction

Cyclic ADP-ribose (cADPR) is a Ca^{2+}-mobilizing cyclic nucleotide active in many cell types and tissues, both in animals, protists and plants (reviewed by Galinoe and Churchill, 2002; De Flora *et al.*, 2004; Guse, 2004a; Lee, 2004). The role of cADPR as an important signal transducer in human cell types implies that analogues of the molecule may become relevant as therapeutical agents in the future.

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Received 22 February 2006; revised 25 May 2006; accepted 14 July 2006; published online 11 September 2006

Accordingly, the biological activity and metabolical stability of a number of such analogues have been studied (reviewed by Guse, 2004b; Potter and Walseth, 2004; Shuto and Matsuda, 2004). Alterations in the adenine base resulted in metabolically stable analogues: 3-deaza-cADPR is a potent and hydrolysis-resistant agonist (Wong *et al*., 1999), whereas 7-deaza-cADPR is also resistant to hydrolysis, but only a partial agonist (Sethi *et al*., 1997). Modifications of the 8-position of adenine usually resulted in antagonists (Walseth and Lee, 1993); thus, further substitution of 7-deaza-cADPR at the 8-position converted the partial agonist 7-deaza-cADPR into the membrane-permeant, hydrolysis-resistant antagonist 7-deaza-8-Br-cADPR (Sethi *et al*., 1997; Guse *et al*., 1999; Schoettelndreier *et al*., 2001). Metabolic stability was also achieved by converting either the 'southern' or the 'northern' ribose into carbocyclic moieties; the resulting analogues cyclic aristeromycin diphosphoribose and cyclic adenosine diphospho-carbocyclic-ribose were biologically active, although at different magnitude (Guse *et al*., 2002), and hydrolysis-resistant (Bailey *et al*., 1996; Shuto *et al*., 2001). Likewise, 2″-amino-cADPR, modified in the 'northern' ribose, was a potent and hydrolysis-resistant agonist (Guse *et al*., 2002).

Despite the wide acceptance of cADPR as a second messenger, its metabolism is still an enigma as the only well-described mammalian enzymes, CD38 and CD157 (reviewed by Schuber and Lund, 2004), catalyse both the formation and breakdown of cADPR. In addition, the active sites of CD38 and CD157 are localized in the extracellular space (or in intracellular vesicles), making a direct involvement of these enzymes in the intracellular metabolism of cADPR difficult. However, to solve this topological paradox, transport systems for the export of nicotinamide adenine dinucleotide $(NAD)^+$ and the import of cADPR have been described in some cell types (Bruzzone *et al*., 2001; Guida *et al*., 2002; Guida *et al*., 2004).

Recently, we have introduced *N*1-cyclic inosine diphosphoribose (*N*1-cIDPR) and a number of its derivatives as mimics of cADPR (Wagner *et al*., 2003; Gu *et al*., 2004; Guse *et al*., 2005; Wagner *et al*., 2005). In the present study, the metabolic stability and the effects of *N*1-cIDPR and some of its derivatives on Ca^{2+} mobilization, cytokine expression and cell proliferation were analysed.

Materials and methods

Drugs and materials

cADPR was obtained from Biolog (Bremen, Germany). NAD was supplied by Roche Diagnostics (Mannheim, Germany). *N*1-cIDPR, 8-bromo-cyclic inosine 5′-diphosphoribose (8-Br-*N*1-cIDPR), *N*1-ethoxymethyl-cIDPR (*N*1-cIDPRE) and *N*1-[(phosphoryl-*O*-ethoxy)-methyl]-*N*9-[(phosphoryl-*O*-ethoxy)-methyl]-hypoxanthine-cyclic pyrophosphate (*N*1-cIDP-DE) were synthesized and characterized spectroscopically as detailed previously (Wagner *et al*., 2003, 2005; Gu *et al*., 2004; Guse *et al*., 2005). ADP-ribosyl cyclase from *Aplysia californica* and NAD glycohydrolase (NADase) from *Neurospora crassa* were purchased from Sigma-Aldrich (Deisenhofen, Germany). NADase was purified as described previously (Gasser *et al*., 2006). All other chemicals used were of the highest purity grade and purchased from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). Culture medium reagents were supplied by Sigma-Aldrich and Gibco-Invitrogen (Auckland, New Zealand).

Jurkat T-lymphocytes cell culture

Jurkat T-lymphocytes (subclone JMP) were cultured as described previously (Guse *et al*., 1993) at 37°C in the presence of 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium containing Glutamax I and *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES) (25 mM) and supplemented with 7.5% newborn calf serum, 100 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin.

Experiments

Metabolic assay and high-performance liquid chromatography analysis of nucleotide degradation. Cyclic nucleotides (50 μM) were incubated with either native CD38 on intact Jurkat T cells (1×10^7, or 2×10^7, or 4×10^7 cells ml^{-1}) in buffer A (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM NaH_2PO_4, 5 mM D-glucose, pH 7.4), or recombinant soluble mouse CD38 (0.75 μg ml^{-1}) in buffer A, or with NADase (180 μg ml^{-1}) in buffer B (1 mM Tris, 2.5 mM $MgCl_2$, pH 7.3) or with ADP-ribosyl cyclase (100 ng ml^{-1}) in buffer C (10 mM NaH_2PO_4, pH 7.3), respectively. Incubation was carried out as indicated at room temperature (RT) under continuous shaking. To control for any background signals in high-performance liquid chromatography (HPLC), intact cells and enzymes were also incubated in the absence of nucleotides under identical conditions. The incubation was stopped by placing the samples into an ice-salt bath. Samples containing intact Jurkat T–lymphocytes were placed into an ice bath, and the cells were rapidly removed by centrifugation for 2 min at 13 700 *g* and 4°C (Heraeus Biofuge fresco). Enzymes were removed from all samples by centrifugation for 30 min at 3000 *g* and 4°C by use of centrifugal filter devices type Centricon YM-10 (10 kDa MW cut-off; Millipore, Bedford, USA).

Reversed Phase-HPLC analysis of nucleotides was performed on a 250 × 4.6 mm Multohyp BDS C18-5μ column (CS Chromatographic Service, Langerwehe, Germany) equipped with a 17 × 4.6 mm guard column filled with the same column material or with a 4.0 × 3.0 guard cartridge containing a C18 (ODS) filter element (Phenomenex, Aschaffenburg, Germany). The separation was performed as described previously (Schweitzer *et al*., 2001) at a flow rate of 1 ml min^{-1} with RP-HPLC buffer (20 mM KH_2PO_4, 5 mM tetrabutylammonium dihydrogen phosphate, pH 6) containing increasing amounts of methanol. The gradient used for separation was (% methanol) 0 min (6.5), 3.5 min (7.5), 5.5 min (16), 8 min (25), 18 min (6.5) and 27 min (6.5). Nucleotides were detected using an UV detector (HPLC detector 432, Kontron Instruments, Neufahru, Germany) at 270 nm for cADPR and at 250 nm for *N*1-cIDPR, 8-Br-*N*1-cIDPR, *N*1-cIDPRE and *N*1-cIDP-DE because of the different absorption maxima of the nucleotides. Integration of peaks was performed with the data–acquisition system MT2 from Kontron Instruments.

Proliferation assay
Jurkat T–lymphocytes (1×10^5 ml^{-1}) were incubated with increasing concentrations of cADPR, *N*1-cIDPR, 8-Br-*N*1-cIDPR, *N*1-cIDPRE or *N*1-cIDP-DE or vehicle in RPMI 1640 medium containing Glutamax I, HEPES (25 mM), 100 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin at 37°C in the presence of 5% CO_2 for 2 h. Then, the medium was supplemented with 7.5% newborn calf serum and the incubation was continued. Cell density was determined after 24, 48, 72 and 96 h with CASY Model DT cell counter (Schärfe System, Reutligen, Germany).

Quantitative PCR
mRNA was extracted using standard protocols (Sigma-Aldrich, Deisenhofen, Germany) and reversed to cDNA (Invitrogen, Auckland, New Zealand). Taqman analysis was performed as reported (Flügel *et al*., 2001) using ABI Prim 7700 Sequence Detector 'Taqman' (PE Applied Biosystems, Foster City, USA). The expression of a housekeeping gene (β-actin) was set into relation to the specific mRNA. Data were obtained by independent duplicate measurements. The C_T value of the individual measurements did not exceed 0.5 amplification cycles.

In vitro *rat cell reactivity assay*
Myelin-basic protein-specific rat T cells retrovirally transfected with green fluorescent protein (GFP) ($T_{MBP-GFP}$ cells) were co-cultured for 48 h in 96-well plates (in Dulbecco's modified Eagle's medium (DMEM) 1% rat serum) with antigen-presenting cells in the presence or absence of specific antigen (10 μg ml^{-1} myelin basic protein (MBP)). Amplification of $T_{MBP-GFP}$ cells was measured by cytofluorometry as described (Kawakami *et al*., 2005). Their numbers were determined in relation to a known absolute number of added phycoerythrin-labelled plastic beads (Becton-Dickinson, Franklin Lakes, USA). The amplification rate was calculated in relation to the GFP^+ T-cell numbers at day 0.

In vitro *human cell reactivity assay*
Human T cells specific for myelin-basic protein were cultured in 96-well plates (in DMEM 7.5% calf serum) in the presence of specific antigen (peptides 139–152 of human myelin-basic protein), or with an irrelevant antigen (tetanus toxoid). [^{3}H] desoxythymin (2 Ci/mmol; Amersham-Buchler, Braunschweig, Germany) was added to the cultures after 24 h. The radioactive label present in the different cultures was determined as described (Flügel *et al*., 2001).

Results

Effects of N*1-cIDPR and its* N*1-coupled cyclic inosine diphosphoribose analogues on cellular Ca^{2+} concentration*
*N*1-cIDPR has recently been shown to mobilize Ca^{2+} from intracellular stores with an effector concentration for half-maximum response (EC_{50}) of approximately 33 μM (Table 1; Wagner *et al*., 2003). The natural nucleotide cADPR gave almost identical results (Table 1; Guse *et al*., 2005; Wagner *et al*., 2005), indicating that the replacement of the imino–group at C6 by an oxo–group does not change binding and interaction properties of the nucleotide at its receptor protein. As both compounds are very polar, only few experiments were conducted with intact cells; as expected, no Ca^{2+} mobilization was observed. 8-Br-*N*1-cIDPR induced a transient Ca^{2+} mobilization in intact cells (Table 1; Wagner *et al*., 2003); however, its Ca^{2+}-releasing activity in permeabilized cells was very low. The latter may indicate that 8-Br-*N*1-cIDPR acts via a mechanism distinct from the one induced by cADPR and is presently under investigation. The compounds *N*1-cIDPRE and *N*1-cIDP-DE were less effective in permeabilized cells as compared to the maximal Ca^{2+} release obtained with cADPR (Table 1; Gu *et al*., 2004; Guse *et al*., 2005). Concentrations of 100 μM for *N*1-cIDPRE and 500 μM for *N*1-cIDP-DE did not even reach the magnitude of Ca^{2+} release obtained by cADPR (Table 1; Gu *et al*., 2004; Guse *et al*., 2005). In intact cells, the EC_{50} for *N*1-cIDPRE was approximately 500 μM (Gu *et al*., 2004), while an exact EC_{50} for *N*1-cIDP-DE could not be determined since at 1 mM extracellular concentration there was still no saturation of the effect (Guse *et al*., 2005); however, it did not appear reasonable to further increase the concentration. Antagonistic effects were not observed with any inosine-based analogues of cADPR (not shown).

Metabolic stability of cADPR against CD38, ADP-ribosyl cyclase and NADase
To study the metabolism of cADPR by CD38, the nucleotide was incubated with intact Jurkat T–lymphocytes. These cells express CD38 as an integral type 2 membrane protein with the active site located in the extracellular space (Da Silva *et al*., 1998). The well-described cADPR-hydrolase activity of CD38 resulted in a slow decrease of cADPR with a concomitant increase in the hydrolysis product adenosine 5′-diphosphoribose (ADPR) (Figure 1a, b and g). ADP-ribosyl

Table 1 Effects of *N*1-cIDPR and some of its derivatives on Ca^{2+} mobilization in Jurkat T cells

Compound	*EC_{50} (μM)*		*Reference*
	Permeabilized cell	*Intact cell*	
cADPR	33	ND	Wagner *et al.* (2005); Guse *et al.* (2005)
*N*1-cIDPR	33	ND	Wagner *et al.* (2005)
8-Br-*N*1-cIDPR	ND	Active	Wagner *et al.* (2003)
*N*1-cIDPRE	>100	Approx. 500	Gu *et al.* (2004)
*N*1-cIDP-DE	>500	>500	Guse *et al.* (2005)

Abbreviations: 8-Br-*N*1-cIDPR, 8-bromo-cyclic inosine 5′-diphosphoribose; cADPR, cyclic adenosine 5′-diphosphoribose; *N*1-cIDP-DE, *N*1-[(phosphoryl-*O*-ethoxy)-methyl]-N9-[(phosphoryl-*O*-ethoxy)-methyl]-hypoxanthine-cyclic pyrophosphate; *N*1-cIDPR, *N*1-cyclic inosine 5′-diphosphoribose; *N*1-cIDPRE, *N*1-ethoxymethyl-cIDPR; ND, not determined.
Permeabilized cells: Jurkat T cells were transferred to an intracellular buffer and permeabilized using saponin as described by Guse *et al.* (2002, 2005) and Gu *et al.* (2004). [Ca^{2+}] was determined ratiometrically in a Hitachi F-2000 fluorimeter in the presence of 1 μM fura2/free acid, ATP (1 mM) and an ATP-regenerating system consisting of creatin kinase and creatin phosphate.
Intact cells: Jurkat T cells were loaded with fura2/AM and analysed by ratiometric fluorimetry in a Hitachi F-2000 fluorimeter as described by Guse *et al.* (2002, 2005) and Gu *et al.* (2004). Extracellular Ca^{2+} was present at 1 mM throughout the experiments.

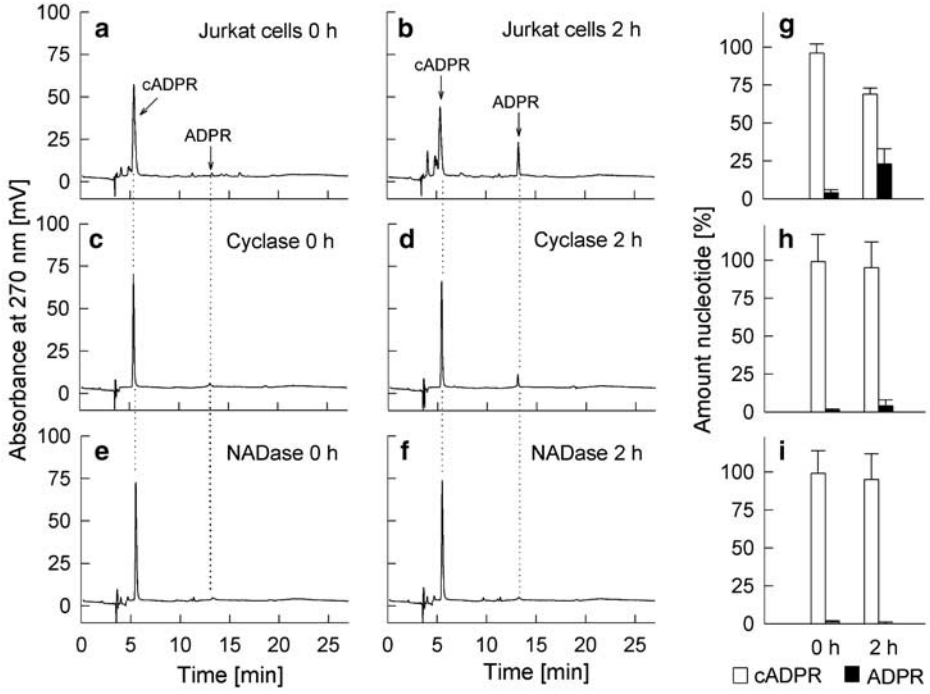

Figure 1 Metabolism of cADPR by CD38, ADP-ribosyl cyclase and NADase. cADPR (50 μM) was incubated with Jurkat T-lymphocytes (1×10^7 ml^{-1}) (**a**, **b**, **g**), ADP-ribosyl cyclase (100 ng ml^{-1}) (**c**, **d**, **h**) and NADase (180 μg ml^{-1}) (**e**, **f**, **i**) for 120 min at RT ($n = 4$–5 each condition). Aliquots were taken at 0 or 120 min and were analysed by RP-HPLC. Characteristic chromatograms are shown. Data in panels g, h and i are mean ± s.d. ($n = 4$–5).

cyclase from *A. californica* displayed only a minor degradation of cADPR (Figure 1c, d and h), although complete cyclization of NAD to cADPR was achieved under the same experimental conditions (data not shown). NADase from *N. crassa* did not metabolize any cADPR under identical conditions (Figure 1e, f and i).

Metabolic stability of N*1-cIDPR against CD38*

As *N*1-cIDPR and some of its analogues displayed Ca^{2+}-mobilizing properties in mammalian cells (Table 1), these compounds are well suited as novel tools for signal–transduction research and may further serve as starting material for the synthesis of novel pharmaceutical compounds. The latter ideally are metabolically stable to maintain a constant effective concentration during therapy.

The stability of *N*1-cIDPR was studied under identical conditions as above for cADPR. As CD38 was the only enzyme capable of hydrolysing cADPR to ADPR to some extent (Figure 1a, b and g), the cyclic inosine nucleotides were incubated with intact Jurkat T cells in a similar manner as for cADPR. Control incubations without substrate were carried out to analyse production and/or release of endogenous nucleotides by intact Jurkat T-lymphocytes (Figure 2a–c). Although slight increases in some compounds were observed, for example, an unknown peak eluting at about 4.5 min and a small amount of adenosine monophosphate, the background produced by the cells during the 18h-incubation period was negligible. The metabolism of cADPR and *N*1-cIDPR, a cyclic nucleotide with a very similar structure (Figure 2d and g) and biological activity as compared to cADPR (Wagner *et al*., 2005), was analysed during incubations with CD38 on intact cells for up to 18 h. Although about 60% of cADPR was metabolized to ADPR within 18 h at cell density of 1×10^7 cells ml^{-1} (Figure 2d–f and j), there was no decrease in the peak area of *N*1-cIDPR (Figure 2g–i and j). Likewise, incubation of cADPR or *N*1-cIDPR for 18 h at room temperature in the absence of intact cells did not result in any significant metabolism (Figure 2j). These experiments in which CD38 expressed on Jurkat T cells was used were confirmed by replacement of the Jurkat cells by mouse recombinant soluble CD38 (Figure 2k). Our data confirm the idea that substitution of the amino/imino–group at C6 by an oxo-group, thus converting the bond between *N*1 and C′1 at the northern ribose into a much more chemically stable, amide bond, produces compounds that are also biologically stable. Further evidence for the biological stability towards CD38 was obtained by increasing the cell density, and thereby the CD38 concentration, during the incubations: while approximately 85% of cADPR was degraded within 6 h when the cell number was increased fourfold, almost no metabolism of *N*1-cIDPR was observed (Figure 2l).

If the amide-like structure of *N*1-cIDPR vs the natural structure of cADPR is indeed the reason for its metabolic stability, one would expect other cyclic inosine derivatives to be similarly stable towards CD38.

*Metabolic stability of 8-Br-*N*1-cIDPR,* N*1-cIDPRE and* N*1-cIDP-DE*

Thus, we next analysed the metabolic stability of 8-Br-*N*1-cIDPR, *N*1-cIDPRE and *N*1-cIDP-DE. These compounds all have an identical amide-like structure as in *N*1-cIDPR, but other parts of the molecule are different, for example, a substitution at the 8-position of the base hypoxanthine, or replacement of the northern ribose or both ribose residues by

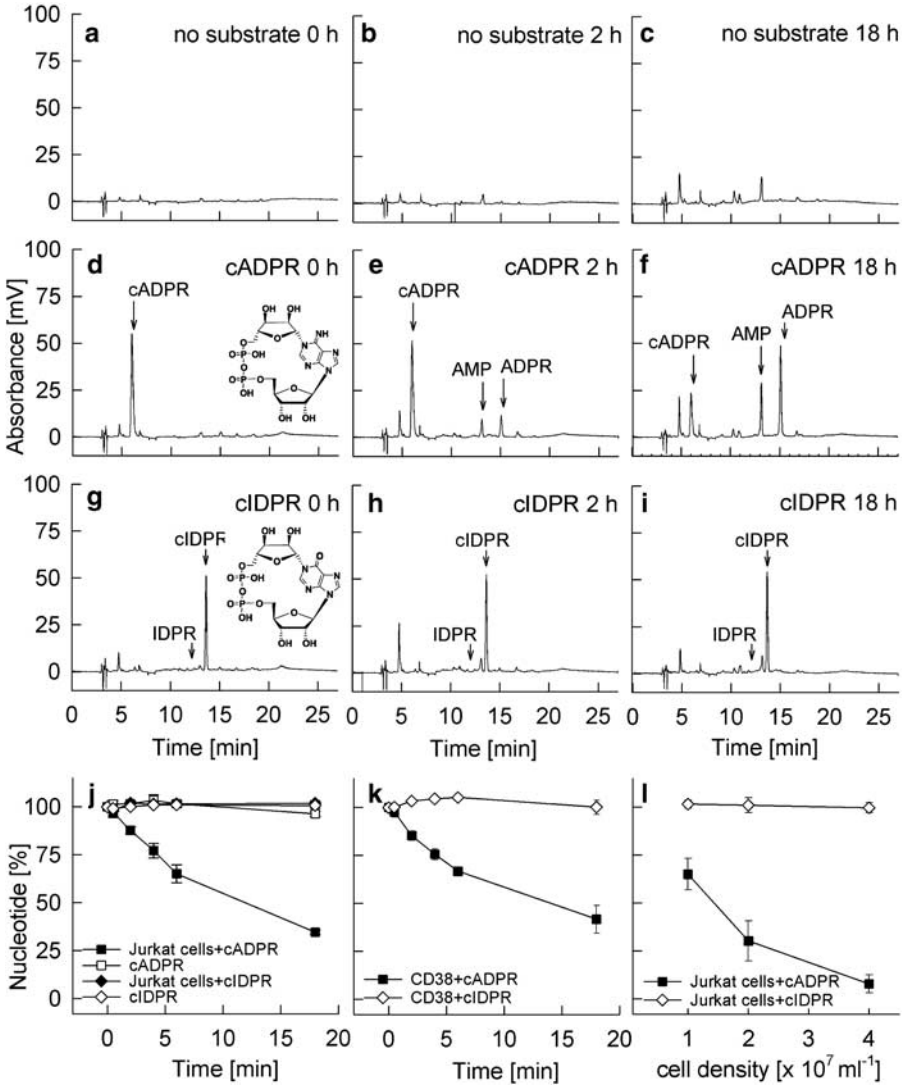

Figure 2 Metabolism of cADPR and *N*1-cIDPR by CD38. Vehicle (no substrate), cADPR (50 μM) or *N*1-cIDPR (50 μM) were incubated at RT either with 1×10^7 ml^{-1} Jurkat T-lymphocytes for the times indicated (**a–i, j**), with recombinant soluble mouse CD38 (0.75 μg ml^{-1}) (**k**) or with increasing numbers of Jurkat T-lymphocytes for 6 h (**l**). Aliquots were taken at the time points indicated and were analysed by RP-HPLC. The detection was performed at 270 nm for cADPR and at 250 nm for *N*1-cIDPR. Data are presented as mean $\pm$ s.d. ($n = 3$). Note that at some time points s.d. values are smaller than symbols and thus cannot be seen properly.

ether bridges (Figure 3). None of the three compounds 8-Br-*N*1-cIDPR, *N*1-cIDPRE and *N*1-cIDP-DE, was metabolized by surface CD38 on intact Jurkat T cells during an 18 h incubation period (Figure 3), suggesting that indeed the amide-like structure present in all inosine-based cyclic analogues of cADPR analysed here is the reason for their stability towards CD38.

All inosine-based cyclic analogues of cADPR described above were also incubated with ADP-ribosyl cyclase (ADPRC) from *A. californica* and NADase from *N. crassa*; however, no degradation of any of the compounds was observed (data not shown).

Cellular effects of cADPR, N*1-cIDPR, 8-Br-*N*1-cIDPR,* N*1-cIDPRE and* N*1-cIDP-DE on T cells*

Finally, cADPR, *N*1-cIDPR and the analogues were incubated with the autonomously proliferating Jurkat T-lymphoma cells. Whereas cADPR and 8-Br-*N*1-cIDPR had no effect on proliferation (Figure 4c), there was a dose-dependent inhibition of proliferation by *N*1-cIDPR (Figure 4a and d), *N*1-cIDP-DE (Figure 4b and d) and *N*1-cIDPRE (Figure 4d).

These antiproliferative effects of *N*1-cIDPR and *N*1-cIDPRE were further studied in primary T cells. Surprisingly, only a very modest and statistically not significant effect on proliferation induced by myelin-basic protein in primary rat T cells was observed (Table 2). Similarly, expression of none of the cytokines, interferon-γ (IFN-γ), transforming growth factor-β, interleukin-2 (IL-2) or the IL-2 receptor, was affected by *N*1-cIDPR or *N*1-cIDPRE, as analysed by quantitative PCR in relation to mRNA of the housekeeping gene β-actin (Table 2). To find out whether the difference observed was due to the different species involved (human Jurkat T cells vs primary rat T cells), a similar experiment was carried out using human myelin-basic protein-specific T cells. Again, at the concentrations of *N*1-cIDPR and *N*1-cIDPRE almost completely blocking autonomous proliferation of Jurkat

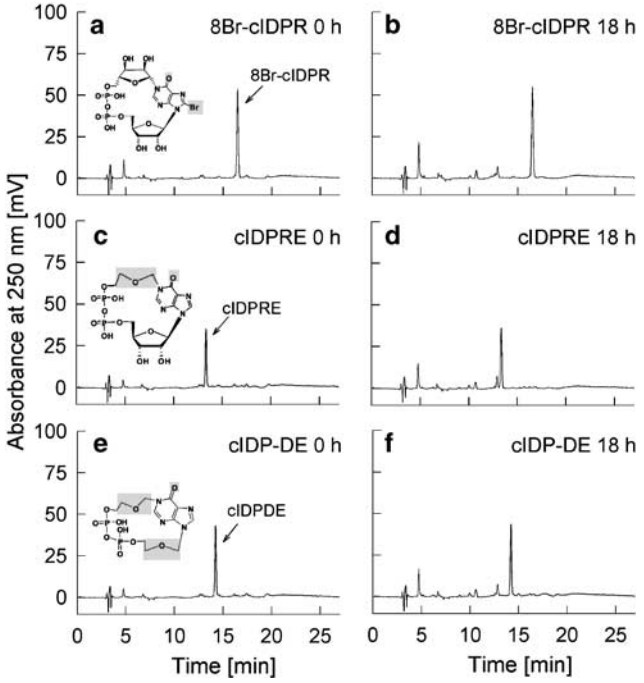

Figure 3 Metabolism of 8-Br-*N*1-cIDPR, *N*1-cIDPRE or *N*1-cIDP-DE by CD38. Jurkat T-lymphocytes (1×10^7 ml^{-1}) were incubated with each 50 μM 8-Br-*N*1-cIDPR (**a**, **b**), *N*1-cIDPRE (**c**, **d**) or *N*1-cIDP-DE (**e**, **f**) for 18 h at RT ($n = 2$). Aliquots were taken at 0 min and 18 h and were analysed by RP-HPLC (detection at 250 nm).

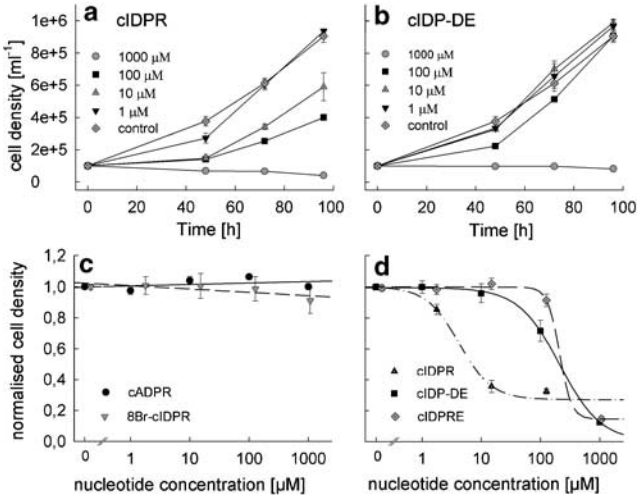

Figure 4 Proliferation of Jurkat T-lymphocytes in the presence of cADPR, *N*1-cIDPR, 8-Br-*N*1-cIDPR, *N*1-cIDPRE or *N*1-cIDP-DE. Jurkat T-lymphocytes (1×10^5 ml^{-1}) were incubated with increasing concentrations of cADPR (**c**), 8-Br-*N*1-cIDPR (**c**), *N*1-cIDPR (**a**, **d**), *N*1-cIDPRE (**d**) or *N*1-cIDP-DE (**b**, **d**) at 37°C/5% CO_2. Cell density was determined after 48, 72 and 96 h. (**a**, **b**) Cell density plotted against incubation time and concentration of *N*1-cIDPR (**a**) and *N*1-cIDP-DE (**b**); data are presented as mean ± s.d. ($n = 3$–9). (**c**, **d**) Concentration–response relationship; data are presented as mean ± s.e. ($n = 2$–3).

T-lymphoma cells, no effect on the proliferation and a slightly stimulatory effect on IFN-γ was observed in the human primary T cells (data not shown).

Discussion

In this report, we describe cellular effects and metabolic stability of the cADPR mimics *N*1-cIDPR, 8-Br-*N*1-cIDPR, *N*1-cIDPRE and *N*1-cIDP-DE.

Jacobson and co-workers were the first to purify a mammalian membrane-bound multifunctional enzyme catalysing both formation of cADPR from NAD, and conversion of cADPR to ADPR (Kim *et al*., 1993). This enzyme was isolated from canine spleen, indicating that leucocytes might express such enzymes. Indeed, the sequence homology between the lymphocyte surface antigen CD38 and ADP-ribosyl cyclase from *A. californica* was first described by States *et al*. (1992); briefly thereafter, Howard *et al*. (1993) described the ADP-ribosyl cyclase and cADPR-hydrolase activity of CD38. However, membrane–bound cADPR-hydrolysing activities were also found in tissue extracts from various invertebrate and vertebrate animals (Lee and Aarhus, 1993), and in human erythrocyte ghosts (Lee *et al*., 1993). Calf spleen NADase was another enzyme displaying cADPR-hydrolase activity (Muller-Steffner *et al*., 1994); interestingly, this enzyme was later identified as bovine CD38 (Augustin *et al*., 2000). Thus, our results using either intact Jurkat T cells or recombinant soluble CD38 confirm the cADPR-degrading activity of CD38 found on the surface of lymphocytes.

Schuber and co-workers described that high concentrations of ADP-ribosyl cyclase from *A. californica* hydrolyzed cADPR to ADPR (Cakir-Kiefer *et al*., 2000); however, as the specificity ratio $V(\max)/K(m)$ was 10 000-fold higher for NAD as compared to cADPR, it is clear that even under our experimental conditions, where complete conversion of NAD to cADPR was observed, only a modest hydrolysis of cADPR was obtained (Figure 1c, d and h).

NADase from *N. crassa* did not metabolize cADPR or any of the *N*1-cIDPR analogues. This finding is consistent with earlier data showing that this enzyme does neither convert NAD to cADPR nor nicotinamide guanine dinucleotide (NGD) to cyclic guanosine 5′-diphosphoribose (cGDPR) (Graeff *et al*., 1994). Thus, it is very unlikely that the *N. crassa* NADase would bind or convert cyclic nucleotides.

The first cyclic inosine derivative published was *N*7-cIDPR (Graeff *et al*., 1996). In contrast to the findings in our current report – metabolic stability of *N*1-cIDPR and its analogues – *N*7-cIDPR was readily hydrolyzed to IDPR by the multifunctional enzyme CD38 (Graeff *et al*., 1996). In addition to *N*7-cIDPR also *N*7-cGDPR was metabolized by CD38, albeit at a lower rate (Moreau *et al*., 2006). The synthesis of the *N*7-cyclized products *N*7-cIDPR and *N*7-cGDPR from NHD and NGD has been explained by a reduced reactivity of *N*1 in case of substitution of the amino/imino-group at C6 by an oxo-group, as exemplified in NHD and NGD. It was proposed that via free rotation of the base around the C1′*N*9 axis from *syn*- to *anti*-position, the more reactive *N*7 was cyclized with C1″ of the northern ribose (Graeff *et al*., 1996). This largely reduced reactivity of the C″1-*N*1 bond in *N*1-cIDPR and its analogues 8-Br-*N*1-cIDPR, *N*1-cIDPRE and *N*1-cIDP-DE very well explains the metabolic stability of these compounds vs CD38-type ADPRC.

Table 2 Effects of *N*1-cIDPR and *N*1-cIDPRE on cytokine expression and proliferation in rat primary T cells

	IFN-γ (rel. copy no.)	*TGF-β (rel. copy no.)*	*IL-2 (rel. copy no.)*	*IL-2 receptor (rel. copy no.)*	*Proliferation (fold increase)*
Control	0.0006±0.0002	0.010±0.0004	0.003±0.0002	0.032±0.025	6.41
MBP alone	0.0019±0.0003	0.014±0.0026	0.005±0.0011	0.046±0.020	20.34
*N*1-cIDPR/MBP	0.0015±0.0005	0.015±0.0016	0.006±0.0006	0.043±0.032	19.75
*N*1-cIDPRE/MBP	0.0017±0.0003	0.011±0.0028	0.004±0.0020	0.058±0.036	18.13

Abbreviations: MBP, myelin basic protein; *N*1-cIDPR, *N*1-cyclic inosine 5′-diphosphoribose; *N*1-cIDPRE, *N*1-ethoxymethyl-cIDPR.
MBP-specific rat T cells were incubated with *N*1-cIDPR (25 μM) or *N*1-cIDPRE (1 mM) in the absence of serum for 2 h. Then, serum and antigen-presenting cells pulsed with MBP were added and the cells were cultured for 48 h. At the 48 h time point, cells were harvested, mRNA was prepared and the number of mRNA copies in relation to the number of mRNA copies of the housekeeping gene β-actin was determined by quantitative PCR. Proliferation was also determined 48 h after stimulation and is expressed as the multiplication factor between time point 0 and 48 h. Data are mean±s.e.m. from two to three independent experiments.

For *N*1-cIDPR and its analogues, our previous study (Wagner *et al*., 2005) confirmed that these novel compounds, which are the closest relatives to the natural cADPR in structure, are chemically very stable. We demonstrated that 8-Br-*N*1-cIDPR is very stable to chemical hydrolysis at normal pH, even at higher temperature. This is likely to be typical of this general class of compound and, unusually, this allowed the direct chemical modification of the 8-position to be undertaken without damage to the rest of the molecule. Moreover, in a recent study we were able to demonstrate that, chemically, the *N*1-amide bond of an 8-halo *N*1-cIDPR is very stable even at low acidic pH, whereas the N9-ribosyl system is degraded giving unusually the N1-inosine-5′-diphosphate ribose derivative that is then further degraded to the mononucleotide (Moreau *et al*., 2006). This is the reverse of what is normally observed for cADPR.

Regarding the effect of inosine-based derivatives on proliferation of Jurkat T cells, some of the results are unexpected. The lack of effect of the membrane-impermeant cADPR was anticipated in Jurkat T cells, although some other cell types have been shown to express transporters for cADPR (Bruzzone *et al*., 2001; Guida *et al*., 2002; Guida *et al*., 2004). Similarly, 8-Br-cIDPR was without effect, although Ca^{2+} mobilization in intact T cells was observed (Wagner *et al*., 2003); however, the fact that the Ca^{2+} signals were only transient in these experiments well explains their lack on proliferation. Both *N*1-cIDPRE and *N*1-cIDP-DE have been shown to evoke large biphasic Ca^{2+} signals in intact Jurkat T cells (Gu *et al*., 2004, Guse *et al*., 2005). In the same concentration range, inhibition of proliferation was observed (Figure 4d), indicating that prolonged Ca^{2+} signalling alone results in cell death. Indeed, initial experiments showed the presence of apoptotic cells in these experiments. This was unexpected as both *N*1-cIDPRE- and *N*1-cIDP-DE-induced Ca^{2+} signalling was in their kinetics similar to Ca^{2+} signalling induced by ligation of the T-cell receptor/CD3 complex (Gu *et al*., 2004; Guse *et al*., 2005). However, it is conceivable that without parallel activation of additional signal–transduction pathways, such as the ras/mitogen-activated protein kinase pathway or the phosphatidylinositol 3-kinase pathway, a situation normally seen upon T-cell receptor/CD3 complex stimulation, Ca^{2+} signalling induced in such an artificial way turns into a death signal. Nevertheless, additional long-term effects of *N*1-cIDPRE and *N*1-cIDP-DE cannot be excluded. Experiments in which 8-Br-cADPR, an established membrane-permeant antagonist of cADPR, was used to revert the inhibitory effects of *N*1-cIDPRE were without success (data not shown). This may, on the one hand, be due to the weak antagonistic effect of 8-Br-cADPR in Jurkat T cells, for example, less than 50% inhibition of cADPR-induced Ca^{2+} release by 100 μM 8-Br-cADPR, or may indicate that the inhibitory effect of *N*1-cIDPRE and *N*1-cIDP-DE on proliferation are mechanistically not linked with Ca^{2+} signalling.

However, the most unexpected result was the strong inhibitory effect of *N*1-cIDPR on Jurkat T-lymphoma cell proliferation (Figure 4a and d). We reasoned that inhibition of ecto-CD38 enzymatic activity by *N*1-cIDPR might be the cause, but initial experiments showed no blockade of the enzymatic activity. Perhaps, *N*1-cIDPR blocks nucleotide pyrophosphatase activity or metabolism of purine monophosphates to the corresponding nucleosides on the surface of the T-lymphoma cells, thereby limiting supply of adenosine and guanosine for the salvage pathway. Whether this indeed is the case will be subject of further investigations. The situation is even more complex as this inhibitory effect of *N*1-cIDPR on autonomously proliferating T-lymphoma cells was not observed in antigen-stimulated primary rat and human T cells (Table 2). Although the underlying mechanism is unclear at present, these results may indicate that *N*1-cIDPR may be used to target specifically T-lymphoma cells without much side effects on primary T cells, even if the latter are proliferating.

In conclusion, although the endogenous second messenger cADPR was degraded by CD38-type ADPRC, *N*1-cIDPR and its analogues were metabolically stable. Along with the proven Ca^{2+}-mobilizing activity of these compounds and the newly discovered inhibitory effects on proliferation of T-lymphoma cells, but not on primary T cells, their metabolic stability is another advantage for the development of new tools for signal-transduction research and potentially also for the design of novel pharmaceutical compounds.

Acknowledgements

This study was supported by the Deutsche Foschungsgemeinschaft (Grant Nos. GU 360/9-1 and 9-2 to AHG), the Deutsche Akademische Austauschdienst (GrantNo. 423/PPP-vrc-sr to AHG, jointly with LHZ), the Gemeinnützige Hertie-Stiftung (Grant No. 1.01.1/04/010 to AHG), the Wellcome Trust (Biomedical Research Collaboration Grant No. 068065 to AHG and BVLP) and the National Natural Sciences Foundation of China (Grant No. SFCBIC 20320130046 and 20132030 to LHZ).

Conflict of interest

The authors have an interest in potential commercial use of the data contained in this publication; thus, a conflict of interest exists.

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