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Quantification of intracellular levels of cyclic ADP-ribose by high-performance liquid chromatography

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Abstract

A combined two-step high-performance liquid chromatographic (HPLC) method was developed for the analysis of endogenous levels of cyclic adenosine diphosphoribose (cADPR) in cell extracts. The detection sensitivity for cADPR was about 10 pmol. Linearity of the HPLC detection system was demonstrated in the range of 10 pmol up to 2 nmol. The method was validated in terms of within-day and between-day reproducibility of retention times and peak areas of standard nucleotides. The method was applied to the analysis of endogenous cADPR in human T cell lines. Sequential separation of perchloric acid extracts from cells on strong anion-exchange and reversed-phase ion-pair HPLC resulted in a single symmetrical peak co-eluting with standard cADPR. The identity of this endogenous material was further confirmed by its ability to be converted to ADPR upon heating the cell samples at 80°C for 2 h. Recoveries of the combined perchloric acid extraction-HPLC analysis procedures were 48.3±10.2%. The determined intracellular concentrations of cADPR in quiescent Jurkat and HPB·ALL human T cells were 198±41 and 28±9 pmol/10^8 cells, respectively. In conclusion, a non-radioactive HPLC method presenting a specificity and sensitivity suitable for precise quantification of cADPR in cell extracts was developed. © 1998 Elsevier Science B.V.

Keywords: ADP; Ribose; Cyclic adenosine diphosphoribose

1. Introduction

Cyclic adenosine diphosphate ribose (cADPR), a metabolite of NAD^+, has recently been shown to induce Ca^{2+} mobilization from intracellular stores in various mammalian and invertebrate cells [1–4]. In addition, the enzymes responsible for the synthesis and hydrolysis of cADPR (ADP-ribosyl cyclase and cADPR-hydrolase, respectively) are widely distributed [5–9], suggesting that cADPR could be a general messenger for mobilizing intracellular Ca^{2+} stores.

We and others have recently shown that cADPR induces Ca^{2+} release from a caffeine-sensitive intracellular store of permeabilized Jurkat and HPB·ALL human T lymphocytes [10,11] and mouse T-lymphoma cells [12].

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Despite its ubiquitous distribution, the precise mechanism of action of cADPR, e.g. whether cADPR functions as a second messenger in response to extracellular stimuli, and the regulation of its activity in living cells are, however, still incompletely understood. Further insights into the signalling function of cADPR are likely to be obtained by direct analysis and quantification of the endogenous levels of this compound in cellular samples.

At present, the method which is most often used for analysis of the cADPR content of tissues is a bioassay based on measurements of the ability of tissue extracts to release Ca^{2+} from another reporter cell preparation, such as sea urchin egg homogenates [13,14]. A few studies on column chromatographic procedures for cADPR analysis have also been described [5,6] but, so far, most of these methods lack the sensitivity and specificity for precise determination of cADPR contents of cells and tissues. In particular, in the case of bioassays, one needs to make sure that, apart from endogenous cADPR, no other Ca^{2+}-release activators and/or inhibitors are present in the sample, which would, otherwise, strongly interfere with the bioassay. As for the column chromatographic methods so far described [5,6], most of the applications included enzymatic assays in which standard cADPR or its precursor were used in relatively high concentrations and, under such conditions, very sensitive detection systems were not an absolute requirement.

More recently, a radioimmunoassay has been described for the analysis of cADPR in tissues [15]. This method was shown to exhibit good sensitivity, but interferences by other nucleotides which cross-reacted with the anti-cADPR anti-serum were observed. In order to improve the specificity of this method, pre-treatment of the samples by treatment with different enzymes was required [15].

In view of this, we have developed an HPLC method for sensitive and specific determination of intracellular levels of cADPR. Applications of this method to assays of endogenous cADPR in human T cell lines are presented. Such a method for purification and analysis of cADPR from cells might set the appropriate conditions for future elucidation of the mechanism by which intracellular levels of cADPR are regulated.

2. Experimental

2.1. Materials

cADPR was either purchased from Amersham-Buchler (Braunschweig, Germany) or was prepared enzymatically from β-nicotinamide adenine dinucleotide (β-NAD^+), as previously reported [16]. 2′-Phospho-cADPR (cADPR-P) was synthesized enzymatically from $NADP^+$, using the *Aplysia* ADP-rybosil cyclase, as previously described [17]. ADP-ribose, NAD^+ and $NADP^+$ were obtained from Sigma (Deisenhofen, Germany). Perchloric acid, methanol (LiChrosolv) and trifluoroacetic acid were purchased from Merck (Darmstadt, Germany). Tetrabutylammonium dihydrogen phosphate was obtained from Fluka (Neu-Ulm, Germany) and Q Sepharose-FF was from Pharmacia Biotech, (Freiburg, Germany). All other chemicals were of the highest purity available. MilliQ water (Millipore Waters, Eschborn, Germany) was used throughout all experiments.

2.2. Cell culture

The human T-lymphocyte cell lines Jurkat and HPB·ALL were cultured as described in earlier publications [10,18].

2.3. Extraction of cADPR from cells

The cells ($1–2\times10^8$ cells per sample) were harvested by centrifugation (450 *g*, 6 min), resuspended in 10 ml of medium containing 140 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgSO_4$, 1 m*M* $CaCl_2$, 1 m*M* NaH_2PO_4, 5.5 m*M* glucose and 20 m*M* Hepes (pH 7.4) and kept at 37°C for 10 min. Endogenous cADPR was extracted from cells using the procedure described by Walseth et al. [13], with some modifications. Briefly, at the end of the incubation period, the cells were pelleted (450 *g*, 2 min) and lysed by addition of 1 ml of ice-cold 3 M $HClO_4$. It is important to note that, from this step on, the extraction procedure was always carried out on 2-ml Eppendorf tubes (Eppendorf-Netheler-Hinz, Hamburg, Germany), since addition of $HClO_4$ to cells or purified cADPR in other types of tubes was found to lead to considerable

losses and degradation of cADPR. The $HClO_4$ extract was immediately Vortex-mixed and frozen twice in liquid nitrogen. After thawing, the samples were left in an ice-salt bath (−5°C) for 30 min to allow for extraction of the soluble nucleotides. Then, precipitated protein was removed by centrifugation (15 000 *g*, 10 min, 4°C), the supernatant was collected and titrated to pH 7–8 by addition of KOH, in order to remove the perchloric acid. After standing for 30 min on ice, the samples were again centrifuged (15 000 *g*, 10 min, 4°C) to remove the $KClO_4$ precipitate. The supernatants containing nucleotides were collected, lyophilized and stored at −80°C. In order to check the recovery of cADPR after freezing in liquid nitrogen, some samples were divided into two identical halves and standard cADPR (1 nmol) was added to one of these half-samples. The subsequent extraction procedure was then carried out on parallel on the two samples. Other agents, namely, trichloroacetic acid (8%, v/v), hydrochloric acid (1 *M*), NaOH (1 *M*), acetic acid (4%, v/v), acetone, methanol (65%, v/v) and ethanol (65%, v/v) were also tested for extracting cADPR from cells, but were found to be less effective and to lead to lower recoveries, as compared to perchloric acid.

2.4. Sample preparation for HPLC analysis

Before analysis on the MiniQ column, ionic contaminants present in the perchloric acid extracts were removed by solid-phase extraction on Q-Sepharose as follows. Disposable columns were filled with Q-Sepharose FF (0.75 ml of wet packed gel), previously brought to the Tris–Cl form. Lyophilized samples were dissolved in a total volume of 40 ml of 1 m*M* Tris (pH 8.5) (conductivity <2 mS) and applied to the column. The columns were washed twice with 2.5 ml of 2 m*M* TFA. Then, the samples were eluted with 5 m*M* TFA (2×2.5 ml), diluted with water to approximately 20 ml and lyophilized.

2.5. HPLC conditions

Two types of HPLC instruments were used – a SMART system (Pharmacia Biotech, Freiburg, Germany) and a Kontron system (Kontron Instruments, Neufahrn, Germany). Analysis of cADPR using the SMART system was carried out as follows. First, a strong anion-exchange HPLC step was performed using a MiniQ PC 3.2/3 column (3-μm particles made of a divinylbenzene–polystyrene co-polymer, 30×3.2 mm I.D., Pharmacia Biotech). Lyophilized cell extracts were dissolved in 1 ml of water and filtered through disposable 0.2-μm filters directly before injection. A gradient from water (later replaced by 1 m*M* Tris, pH 8.0) to 150 m*M* TFA was used at a flow-rate of 400 μl/min. The gradient was (in % of eluent B): 0 min, 0%; 5 min, 0%; 7.5 min, 2.3%; 11 min, 2.3%; 17 min 25%; 18 min, 25%; 18.5 min, 0%; 25 min, 0%. Absorbance was measured at 254 nm and material co-eluting with standard cADPR was collected, lyophilized and redissolved in 100 μl of buffer for reversed-phase ion-pair HPLC (25 m*M* KH_2PO_4, 0.04% tetrabutylammonium hydrogen sulphate, pH 4.0). A reversed-phase column C_2/C_{18} SC 2.1/10 (5 μm; 100×2.1 mm I.D.; Pharmacia Biotech) was used with a gradient from reversed-phase ion-pair buffer to methanol at 200 μl/min. The gradient was (in % of methanol): 0 min, 0%; 1.5 min, 0%; 4.5 min, 3.2%; 6.5 min, 3.2%; 20 min, 20%; 21 min, 20%; 22 min, 0%.

HPLC analysis of cADPR using the Kontron system was performed in the following way. The first, strong anion-exchange HPLC step was carried out using a PRPX 100 matrix (10 μm, Hamilton, Switzerland) packed into a 250×4.6 mm I.D. PEEK column (CS Chromatographie Services, Germany). Other types of columns, namely MiniQ PC 3.2/3 and MonoQ HR 5/5 (Pharmacia, Biotech) were also tested, but were found to have lower performance in comparison to the PRPX 100 column (in terms of selectivity, sensitivity and peak width). Samples were dissolved in 1 ml of 1 m*M* Tris–HCl buffer, pH 8.0, and filtered through disposable 0.2-μm filters directly before injection. An autosampler (model 360, Kontron Instruments) coupled to the HPLC system was routinely used for injection of samples. A gradient from 1 m*M* Tris, pH 8.0, to 150 m*M* TFA was used. Optimal flow-rate was 1.5 ml/min and the gradient was (in % of eluent B): 0 min, 5%; 5 min, 5%; 12 min, 21%; 13 min, 21%; 25 min, 50%; 26 min, 5%; 29.5 min, 5%.

Material co-eluting with standard cADPR was also collected, lyophilized and redissolved in 200 μl of reversed-phase ion-pair HPLC buffer (20 m*M* KH_2PO_4, 5 m*M* tetrabutylammonium hydrogen phosphate). The reversed-phase HPLC column used was a Hypersil BDS C_{18} column (5 μm; 250×4.6 mm I.D.; Hypersil, Cheshire, UK). Other columns tested with this system were a μRPC C_2/C_{18} SC 2.1/10 column (Pharmacia Biotech) and a TSK-gel SuperODS column (2 μm particle size, 100×4.6 mm I.D.; TosoHaas, Stuttgart, Germany). A gradient from reversed-phase ion-pair buffer (see above) to methanol was used at a flow-rate of 1.0 ml/min. The gradient was (in % of methanol): 0 min, 5%; 3 min, 15%; 8 min, 50%; 16 min, 50%; 18 min, 5%; 21 min, 5%.

The UV-absorbance detector (model 432, Kontron Instruments) was autozeroed at the start of each chromatogram and absorbance was measured at 270 nm. Data were processed by the MT2 data acquisition system from Kontron Instruments.

2.6. Heat-inactivation of endogenous cADPR

In some experiments, after chromatography of perchloric acid extracts from Jurkat cells on the strong anion-exchange column, material co-eluting with standard cADPR was collected, lyophilized and redissolved in water (200 μl). This sample was then divided into two and one half-sample was incubated at 80°C, for 2 h, in order to convert endogenous cADPR into ADPR. Subsequently, each sample was analysed on the reversed-phase HPLC system, as described above.

3. Results and discussion

3.1. HPLC analysis of cADPR-development and validation of the method

The aim of the present work was to develop a specific and sensitive method for the analysis and quantification of cADPR and related compounds in cellular samples. As mentioned in the Section 2, in order to establish such a method, two types of HPLC instruments (SMART system, Pharmacia and Kontron system, Kontron Instruments), as well as different types of HPLC columns and buffer conditions, were tested. The performance of the various experimental setups was evaluated and compared with respect to selectivity, sensitivity, peak width, peak symmetry and speed of separation.

Irrespective of the type of HPLC instrumentation used, the method developed is based on two sequential HPLC steps: first, a strong anion-exchange HPLC step followed by reversed-phase ion-pair chromatography.

With the SMART system initially employed, the first HPLC step was carried out using a MiniQ PC 3.2/3 column, while the second chromatography was performed on a C_2/C_{18} SC 2.1/10 micro-reversed-phase column. Under the experimental conditions used, we were able to obtain a broad peak, co-eluting with standard cADPR, in samples from Jurkat T-cells. However, the fact that the SMART system needs a delay time for refilling of the pump cylinders between individual runs resulted in reduced reproducibility of retention times and peak areas of cADPR, especially if reversed-phase columns were used (standard deviations for retention time and peak area of cADPR using the MiniQ column were 1.4 and 7.8%, respectively, while the corresponding values with the reversed-phase column were 7.0 and 11.0%).

As an alternative to this type of methodology, a Kontron system based on a different principle of operation of the pumps (classical two-piston high-pressure pumps) was subsequently tested and is now routinely used for analysis of cADPR. With this instrumentation, three types of strong anion-exchange HPLC columns were initially tested: MiniQ PC (Pharmacia), MonoQ HR 5/5 (Pharmacia) and PRPX 100 (CS Chromatographie Service) columns. Fig. 1 shows typical chromatograms from Jurkat cell extracts obtained with each of these columns. Separation of standard nucleotides and, in particular, of cellular components was considerably more efficient on the PRPX 100 column (Fig. 1C) as compared with the two other types of columns used (Fig. 1A,B). Therefore, this column was selected for all subsequent experiments.

After chromatography of Jurkat cell extracts on the PRPX 100 column, a broad peak co-eluting with standard cADPR was obtained (Fig. 1C). Further analysis and characterization of this material was

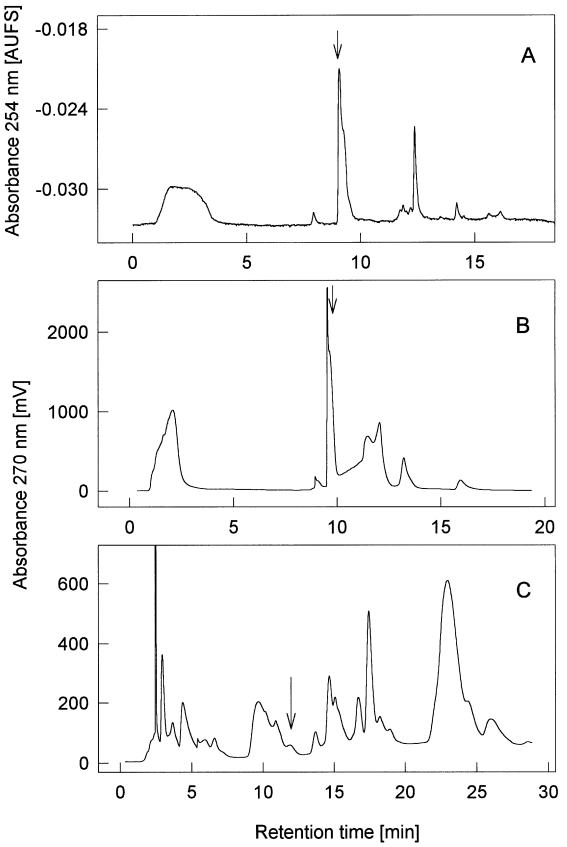

Fig. 1. Selection of a strong anion-exchange column for HPLC analysis of cADPR in cell extracts. A perchloric acid extract from Jurkat T cells ($5–10\times10^7$ cells) was analysed by HPLC on a (A) MiniQ PC 3.2/3 column, (B) MonoQ HR 5/5 column and a (C) PRPX100 column, using the gradients and the experimental conditions described in Section 2. Each chromatogram is representative of at least 15 independent experiments. The arrows indicate the retention time of standard cADPR.

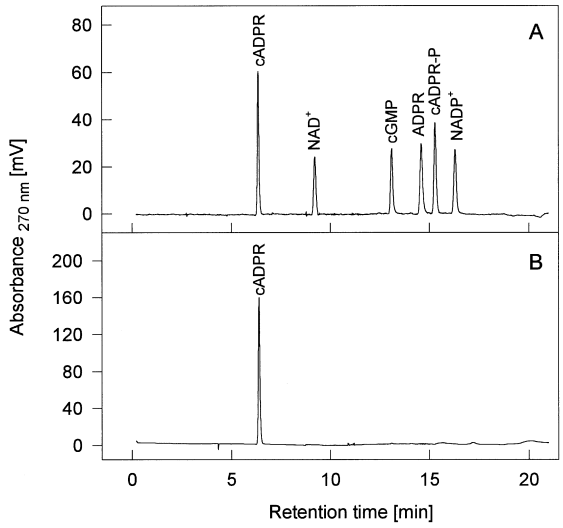

Fig. 2. HPLC analysis of a mixture of standard nucleotides. A mixture of the indicated nucleotides was analysed on a reversed-phase Hypersil BDS C_{18} column, using a gradient from reversed-phase ion-pair buffer (20 m*M* KH_2PO_4, pH 6.0, 5 m*M* tetrabutylammonium hydrogen phosphate) to methanol (see Section 2), at a flow-rate of 1.0 ml/min. (A) Mixture of nucleotides, 500 pmol of each compound; (B) standard cADPR, 1.0 nmol. The chromatograms are representative of at least 10 independent experiments.

carried out by collecting, lyophilizing and subsequently injecting the peak on a second, reversed-phase HPLC column. Two different reversed-phase columns were also tested with the Kontron system. A TSK-gel Super-ODS column was initially used but, with prolonged usage, the performance of this column was considerably reduced and doubling of peaks was often observed. More recently, a silica-based reversed-phase Hypersil column has been tested and found to be the method of choice for the analysis of cADPR. Fig. 2 shows typical chromatograms obtained for a mixture of different nucleotides, demonstrating the good resolution of this type of column. This method was validated with respect to variations of retention time and peak area of standard cADPR (1 nmol) in spiked cell samples, measured either on the same day or over a period of several days. The standard deviation of the retention time of cADPR was 2.3% when analysed on different days, over a period of 2 months, and 1.3% when analysed on the same day. Peak area measured on the same day showed a standard deviation of 2.8%, while the corresponding standard deviation analysed over a period of 2 months was 3.8%.

Linearity was demonstrated by analysing peak areas obtained for cADPR alone or in spiked cell samples containing different amounts of this nucleotide. The calibration curves obtained for cADPR showed a quasi-linear relationship between peak area and amount of compound injected in the range 10 pmol–2.0 nmol, demonstrating that quantitation of this nucleotide in cellular samples is possible by the HPLC method described. The linear regression line and the correlation coefficient for cADPR alone were: area (mV·min)=13.5 amount cADPR (nmol)−

0.05 and $r=0.9997$. The corresponding parameters for cADPR in spiked cell samples were: area (mV·min)=7.46 amount cADPR (nmol)+0.12 and $r=0.9998$.

The detection limits (defined as a 3:1 signal-to-noise ratio) were: for cADPR 10 pmol, for ADPR 15 pmol, for NAD^+ 40 pmol, and for cADPR-P 15 pmol. Such detection limits allow the analysis of biological samples, e.g. primary cell cultures, tissue samples, body fluids, typically containing minute amounts of cADPR and some related nucleotides (see Section 3.2). Except for a published radioimmunoassay [15], the threshold value of detectability for cADPR using the present HPLC method represents also an improvement, when compared to the sensitivities reported earlier, with other methodologies [5,6,13].

3.2. HPLC analysis of cADPR – applications

Application of the combined two-step HPLC method to the analysis of Jurkat and HPB·ALL cell extracts resulted in chromatograms of the type represented in Fig. 3. Upon separation on the strong anion-exchange column, cell samples were resolved into several relatively broad peaks, one of which co-eluted with standard cADPR (Fig. 3A,D). Taking into account that small differences in retention times might occur, identification of the peak which would contain endogenous cADPR was confirmed both by co-chromatography of the cell sample with standard cADPR and by analysis, in parallel, of cell samples to which the standard nucleotide was initially added during the extraction procedure.

Subsequent separation of the collected, cADPR-containing material by reversed-phase ion-pair HPLC resulted in a single, sharp and symmetric peak co-eluting with standard cADPR (Fig. 3B,E). As described for the first HPLC step, co-chromatography of the cell sample with standard cADPR, as well as analysis of cell samples to which the standard nucleotide was added during the extraction procedure, also confirmed the identification of the endogenous cADPR peak (Fig. 3C,F).

The recovery of the complete analytical method was evaluated by quantification of the cADPR in 'twin' samples, one of which was previously spiked with a known amount of standard cADPR. This

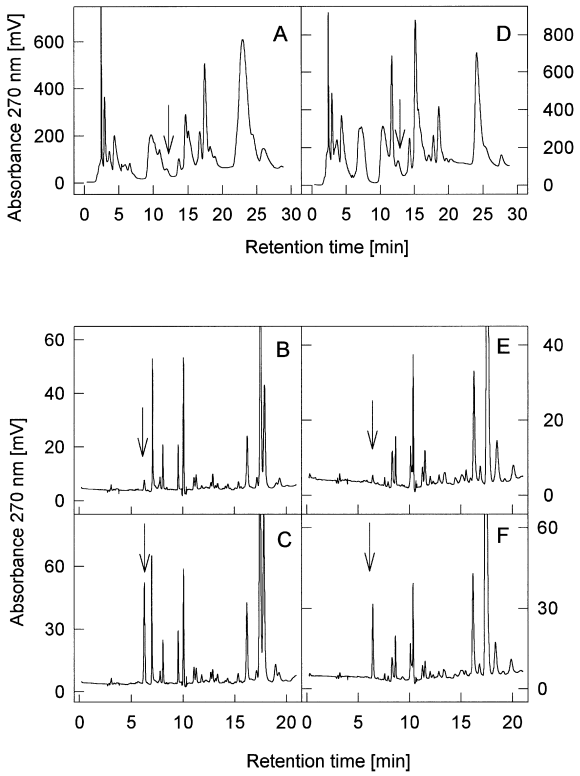

Fig. 3. HPLC analysis of endogenous cADPR in Jurkat and HPB·ALL T cells. A perchloric acid extract from Jurkat cells (8×10^7 cells; A–C) or HPB·ALL cells (1×10^8 cells; D–F) was analysed by (A,D) strong anion-exchange HPLC on a PRPX 100 column using a gradient from 1 m*M* Tris, pH 8.0, to 150 m*M* TFA at a flow-rate of 1.5 ml/min. Material co-eluting with standard cADPR was collected, lyophilized and subsequently analysed by (B,E) reversed-phase ion-pair HPLC on a Hypersil column, using the conditions described in Section 2. Co-chromatography of cellular material with standard cADPR (0.5 nmol) on reversed-phase HPLC gave chromatograms of the type shown in (C,F). The retention time of standard cADPR is indicated by the arrow. The tracings are representative of at least eight independent experiments.

procedure led to recovery values of 48.3±10.2%. These values are in the range of previously reported recovery data, using similar cADPR extraction procedures. In contrast, the higher recoveries reported by Takahashi et al. [15] and by Malaisse et al. [19], using 4% trichloroacetic acid for extraction of cADPR could not be reproduced; using such procedure we obtained much less effective extraction of cADPR from cells (recoveries in the range of 10%), as compared to the protocol with 3 *M* perchloric

acid. However, it should be mentioned that ATP was the reference compound used by those authors [19] for calculating recovery and no correction was made for recovery of cADPR. Such methodology represents a gross simplification and must lead to inadequate results, because the chemical stability of cADPR is different from that of ATP (see also Fig. 4A).

The calculated effective concentrations of cADPR in the Jurkat and HPB·ALL cell lines studied, after correcting for the recovery of our extraction-analysis method, are shown in Table 1. Jurkat T cells showed higher intracellular levels of cADPR (198±41 pmol/10^8 cells), as compared to HPB·ALL T cells (28±9 pmol/10^8 cells). For comparison, Table 1 also indicates the concentrations of cADPR previously determined in these cell lines by a less-sensitive and -specific HPLC method [11]. Under those conditions, relatively high amounts of cADPR were detected in Jurkat and in HPB·ALL T cells. However, as discussed in this earlier publication [11], due to the lower resolution of the HPLC columns used and, therefore, the possibility that other compounds with similar physicochemical properties might co-elute with cADPR, a potential overestimation of those endogenous cADPR concentrations was considered. With the presently developed HPLC method, the cellular material which was previously attributed to cADPR could be further resolved into several additional peaks. Therefore, a considerable improvement in terms of sensitivity has been achieved with our new HPLC method.

As a further approach to validate the HPLC method, the identity of the endogenous material co-eluting with standard cADPR on the reversed-phase Hypersil column was confirmed by heat-inactivation and conversion to ADPR (Fig. 4). Control experiments showed that incubation of standard cADPR (1 nmol) at 80°C, for 2 h, resulted in complete conversion of the cyclic nucleotide to ADPR (Fig. 4A). When the cellular material identified as endogenous cADPR was subjected to this same type of treatment and re-chromatographed on the reversed-phase column, the peak corresponding to cADPR was no longer observed on the chromatograms; instead, a new peak with the same retention time as standard ADPR was obtained (Fig. 4B,C). Moreover, the area of this ADPR peak was similar to the area of the

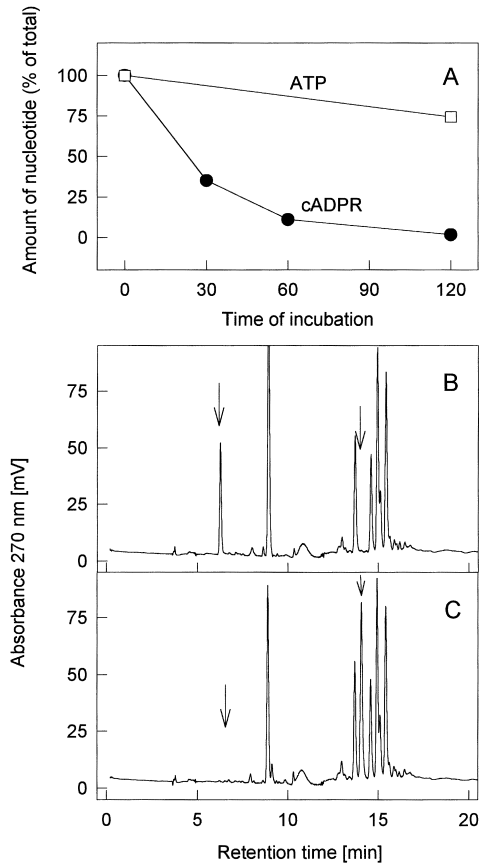

Fig. 4. Heat-inactivation of endogenous cADPR. A perchloric acid extract from Jurkat cells (8×10^7 cells plus internal standard cADPR, 0.5 nmol) was analysed by strong anion-exchange HPLC, as described in Section 2. The material co-eluting with standard cADPR was collected, lyophilized, redissolved in water and subsequently incubated at 80°C, for 2 h. The heat-inactivated cellular material was then chromatographed on the reversed-phase HPLC system. (A) Time course of conversion of standard cADPR into ADPR (closed symbols) and of standard ATP (opened symbols), upon heating at 80°C. Data is represented as mean of three independent experiments. (B) Reversed-phase chromatogram from a control, non-heated cell sample. (C) Reversed-phase chromatogram from a heat-inactivated cell sample. The retention times of standard cADPR (6.2 min) and ADPR (14.5 min) are indicated by the arrows. Material eluting at 9.5 min corresponds to NAD^+. The chromatograms are representative of three independent experiments.

previously detected cADPR peak on non-heated samples (a partial conversation of NAD^+ to ADPR upon heating was also observed, which accounted for the slightly higher ADPR-peak area, as compared to the area of the cADPR peak). This result provides

Table 1
Endogenous levels of cADPR in human T cell lines

Cell line	Concentration of cADPR (pmol/10^8 cells), mean±S.D.	Previously measured [cADPR] (nmol/10^8 cells)[a]
Jurkat	198.0±41.1 (n=10)	3.08±0.19 (n=16)
HPB·ALL	28.2±9.0 (n=8)	4.37±0.12 (n=4)

The concentration of cADPR in the human cell lines was determined with the combined two-step microbore-HPLC method described in the text. The intracellular concentrations of cADPR are given as mean±S.D., after being corrected for recovery. For comparison, the concentrations of cADPR previously determined in these T cell lines by a less-sensitive and -specific HPLC method are also shown.
[a]Guse et al. [11].

further strong evidence and confirms the identity of the cADPR-peak shown on our chromatograms.

4. Conclusions

(1) A non-radioactive, sensitive and specific HPLC method has been developed for quantification of endogenous cADPR in cellular samples. With a detection limit of a few picomoles, this method is suitable for measuring intracellular levels cADPR.

(2) Co-elution on strong anion-exchange, as well as reversed-phase HPLC and heat-inactivation of the HPLC peak attributed to endogenous cADPR, confirmed the identity of the compound.

(3) The established HPLC method may set the appropriate conditions for further elucidation of the mechanism by which intracellular levels of cADPR are regulated in response to extracellular stimuli and, in this way, may contribute to clarify the putative second messenger role of cADPR.

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