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Journal of Chromatography B, 821 (2005) 181–187
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of intracellular concentrations of the TRPM2 agonist ADP-ribose by reversed-phase HPLC

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Received 1 March 2005; accepted 2 May 2005
Available online 25 May 2005

Abstract

Since the NAD metabolite ADP-ribose (ADPR) has recently gained attention as a putative messenger, a method was established for the quantification of intracellular ADPR by reversed-phase HPLC. Cellular nucleotides were extracted with trichloroacetic acid, and crude cell extracts purified by solid phase extraction using a strong anion exchange matrix. After optimization of the extraction procedure, cellular ADPR levels were determined using two different reversed-phase columns (C18 versus C12), operated in ion pair mode. Intracellular ADPR concentrations in human Jurkat T-lymphocytes and murine BW5147 thymocytes were determined to be 44 ± 11 μM and 73 ± 11 μM, respectively.

Keywords: ADP-ribose; TRPM2; K_{Ca} channels; HPLC; Calcium signaling; Solid phase extraction

1. Introduction

During the last years ADP-ribose (ADPR), a metabolic product of nicotinamide adenine dinucleotide (NAD) or of poly(ADP-ribose), has gained attention as a putative intracellular messenger, acting on different plasma membrane cation channels. First, a modulatory effect of ADPR on Ca^{2+}-activated K^+ (K_{Ca}) channels in excised inside-out patches from coronary arterial smooth muscle cells was demonstrated [1]. The presence of at least 0.1 μM ADPR in the bath solution, equivalent to the cytosol, dose-dependently increased the open state probability of K_{Ca} channels without altering the amplitude of the currents [1].

Stimulation of smooth muscle cells by the vasoconstricting agonist 11,12-epoxyeicosanotrienoic acid resulted in increase NAD glycohydrolase (NADase) activity; moreover, vasoconstriction was sensitive to inhibition of NADase [2]. Therefore, ADPR was supposed to be a signaling molecule mediating the 11,12-EET induced activation of K_{Ca} channels [2].

More recently, ADPR was also revealed as an agonist of the cation channel TRPM2 [3–5], which is a non-selective, Ca^{2+} permeable plasma membrane channel, belonging to the transient receptor potential (TRP)-family of ion channels (reviewed in [6,7]). In contrast to K_{Ca} channels, TRPM2 is gated by much higher cytosolic concentrations of ADPR (EC_{50} 90 μM; refs. [3,4]). Although TRPM2 is supposed to be involved in cellular Ca^{2+} signaling [8], the physiological role of its agonist ADPR is still unclear, since intracellular concentrations in cells expressing TRPM2 have not been previously reported. Therefore, it is of crucial importance to develop a method for the extraction and quantification of the ADPR levels actually present in cells, since this will give insights into physiological functions of this compound and possibly reveal its role in cellular signaling.

Different approaches have been used for the analysis of cytosolic nucleotides and related molecules like NAD or NADP [9–14], but none of these methods can be easily adopted to quantify cellular ADPR levels. Recently, we developed

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doi:10.1016/j.jchromb.2005.05.002

HPLC methods to determine the second messenger cyclic ADP-ribose (cADPR) [15,16], and to analyze enzymatic ADP-ribosyl cyclase activity [17]. Since most ADP-ribosyl cyclases are bi-functional and possess both ADP-ribosyl cyclase and NADase activity [18], they may also be involved in the enzymatic formation of ADPR. In the present study, the HPLC technique for determination of cADPR was used as starting point to develop an analytical method for the quantification of intracellular ADPR levels in two lymphatic cell lines.

2. Materials and methods

2.1. Materials

Nucleotide standards were purchased from Sigma (Deisenhofen, Germany) or from Boehringer (Mannheim, Germany). Trichloroacetic acid (TCA), diethyl ether, methanol (LiChrosolv) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Tetrabutylammonium dihydrogenphosphate (TBAHP) was obtained from Fluka (Neu-Ulm, Germany) and Q-Sepharose FF was from Amersham Biosciences (Freiburg, Germany). All other chemicals were of the highest purity available. SeralPur water (Seral, Ransbach, Germany) was used during all experiments.

2.2. Cell culture

The human T-lymphocyte cell line Jurkat (clone JMP) was cultured in RPMI 1640 with Glutamax I, supplemented with 7.5% (v/v) newborn calf serum (Biochrom, Berlin, Germany), 100 U/ml penicillin, 50 μg/ml streptomycin and 25 mM HEPES, pH 7.4. The density of the cells was adjusted to 0.2–1.0 × 10^6 cells/ml. The murine thymoma cell line BW5147 was cultured as described for Jurkat cells with some modifications: The medium was additionally supplemented with MEM non-essential amino acids (Invitrogen, Groningen, Netherlands), and the cells were diluted to a density of 0.1 × 10^6 cells/ml with fresh medium every second day. All cells were kept at 37 °C in a humidified incubator at 5% (v/v) CO_2 in air. Counting of cells and determination of the cell volumes were performed using a CASY TT 1 system (Schärfe System, Reutlingen, Germany).

2.3. Extraction of intracellular ADPR

The cells (5 × 10^7 cells for Jurkat cells, 1 × 10^8 cells for BW5147 cells) were harvested by centrifugation (550 × *g*, 5 min, room temperature), resuspended in 5 ml of buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM NaH_2PO_4, 5.5 mM glucose, 20 mM HEPES, pH 7.4, and kept at 25 °C for 30 min. Endogenous ADPR was extracted using the following procedure: at the end of the incubation the cells were collected as above and resuspended in 2 ml ice cold 20% (w/v) TCA. The samples were then rapidly frozen in liquid nitrogen and thawed at 37 °C for three times, lysed with an ultrasonic disruptor for 20 s (Bandelin Sonoplus UW70, Berlin, Germany), and cell debris was removed by centrifugation (550 × *g*, 5 min, 4 °C). The supernatants were collected and divided into two identical halves (twin-samples), to one of which 2.5 nmol ADPR was added to identify the ADPR peak in the chromatogram and to calculate the recovery during the extraction procedure. The samples were left on ice for 30 min to allow the extraction of cellular nucleotides, and then centrifuged two times at 13,000 × *g* for 10 min at 4 °C to remove precipitated proteins. The supernatants (about 1 ml) were collected and extracted three times with 5 volumes of water-saturated diethyl ether by Vortex-mixing for 1 min and removing the ether phases. The samples were lyophilized for 60 min to remove traces of ether and stored at −70 °C. In some experiments, the neutralized extracts of 5 × 10^6 cells were directly used for HPLC analysis. Artificial samples consisted of 100 μl BSA solution (100 mg/ml). To some of these samples, 20 nmol NAD and/or 20 nmol ADPR were added. The extraction procedure was performed in the same way as described above for samples consisting of cells.

2.4. Purification of cell extracts by solid phase extraction

For solid phase extraction of neutralized TCA extracts, plastic filtration tubes (Supelco, Bellefonte, USA) were packed with 250 μl Q-Sepharose FF and were cleaned in place with 5 ml 150 mM TFA. Then, columns were equilibrated with 15 ml 10 mM Tris–HCl, pH 8.0. The cellular extracts were diluted to a volume of 14 ml with 10 mM Tris–HCl, pH 8.0 and applied to the columns. The columns were then washed first with 30 ml 10 mM Tris–HCl, pH 8.0 followed by 600 μl 5 mM TFA. For the elution of ADPR, 1 ml of a 20 mM TFA solution was applied in aliquots of 100 μl. It is important to note that the eluates were collected in glass vials, since the use of polypropylene tubes resulted in problems with degradation of the samples, obviously by compounds stemming from the tube walls. The samples were stored at −70 °C and analyzed by HPLC as soon as possible.

2.5. HPLC conditions

HPLC analysis of endogenous ADPR using a Kontron HPLC system was performed in the following way: The eluates of the solid phase extraction were filtered through disposable 0.2 μm filters (Sartorius, Göttingen, Germany) directly before injection. Eighty microlitres of the samples (corresponding to the extract of 2 × 10^6 cells or 4 × 10^6 cells for Jurkat and BW5147 cells, respectively) were mixed with 20 μl buffer containing 100 mM KH_2PO_4, 25 mM TBAHP, pH 6.0. An autosampler (model 360, Kontron Instruments) was routinely used for the injection (100 μl injection volume) of samples and standards. The reversed-phase HPLC columns used were either a Multohyp BDS C18 column

(250 mm × 4.6 mm, particle size 5 μm) protected by a guard column (17 mm × 4.6 mm) of the same material (both Chromatographie Service, Langerwehe, Germany) or a Synergi MAX RP 80A C12 column (250 mm × 4.6 mm, particle size 4 μm) protected by a SecurityGuard device (Phenomenex, Aschaffenburg, Germany). For both columns a gradient from reversed-phase ion-pair buffer (20 mM KH_2PO_4, 5 mM TBAHP, pH 6.0) to methanol was used at a flow rate of 1.0 ml/min. The gradient contained in percent of methanol: 0 min 15, 3.5 min 15, 5.5 min 32.5, 6.5 min 32.5, 9 min 40, 11 min 50, 16 min 50, 18 min 15, 27 min 15. The UV detector (model 432, Kontron Instruments) was autozeroed at the start of each chromatogram and absorbance was measured at 270 nm. To further investigate the ADPR peak in cell extracts, the flow was stopped during the presence of this peak in the flow cell of the UV detector, and the wavelength was adjusted from 210 nm to 340 nm using steps of 10 nm. The absorption at each wavelength was corrected for the background obtained from blank samples, and the results compared between spectra obtained from either cell extracts or genuine ADPR. Data were processed by the MT2 data acquisition system from Kontron Instruments. The recovery of the extraction was calculated by comparing ADPR standards with spiked and non-spiked samples.

3. Results

A HPLC based method for the determination of the second messenger cADPR [15] was used as starting point to develop a method to quantify cytosolic ADPR. The analysis of ADPR by reversed-phase HPLC was first established using nucleotide standards. To examine the separation of ADPR from other soluble compounds present in cell extracts, a panel of 15 nucleotide standards was analyzed using a Multohyp BDS C18 reversed-phase column, operated in ion-pair mode (Fig. 1). All components of the standard mixture were clearly separated, except the pairs AMP/nicotinic acid and 2′-phospho-ADPR/nicotinic acid adenine dinuleotide phosphate (NAADP). Importantly, ADPR did not co-elute with any of the tested compounds. To test linearity and detection limit of the quantification by UV absorbance, different amounts of standard ADPR were separated and the peak areas were calculated. Quantification of ADPR by UV absorption was linear up to 50 nmol ($r^2 = 0.99$, $n = 9$ for each of six points of calibration). Intra- and inter-day precision of the retention times of ADPR were 11.41 ± 0.02 min ($n = 18$, mean ± S.D.) within one day and 11.38 ± 0.08 min ($n = 54$, mean ± S.D.) within one week. The detection limit for standard ADPR was 5 pmol at a signal/noise ratio of >3.

After having optimized the separation of ADPR with standards, a method for the extraction of intracellular ADPR was established. A protocol using cell lysis by freeze–thaw-cycles in the presence of 20% (w/v) trichloroacetic acid (TCA) was chosen due to the immediate acidic denaturation of cellular compounds, terminating all metabolic reactions [9]. However, the direct HPLC analysis of the crude cell extracts after removal of TCA by extraction with diethyl ether resulted in chromatograms of decreasing quality (Fig. 2B–D). Upon repeated analysis of the same extract, a rapid drop in separation performance was observed, e.g. occurrence of asymmetrical peaks and peak broadening of the ADPR peak and a peak eluting directly before (Fig. 2, insets in B–D); similar problems were observed in all regions of the chromatograms (Fig. 2, arrowheads). Also an increase of the column backpressure occurred due to clogging of the HPLC columns. Therefore, the direct analysis of the neutralized cell extracts was not suitable for determination of ADPR. For this reason, a solid phase extraction using a strong anion exchange matrix (Q-sepharose FF) was additionally carried out before HPLC analysis of the extracts. The TFA eluates of the solid phase extraction column were then directly analyzed by reversed-phase HPLC. The additional solid phase extraction apparently solved the problems with column clogging and reduced separation performance (see below).

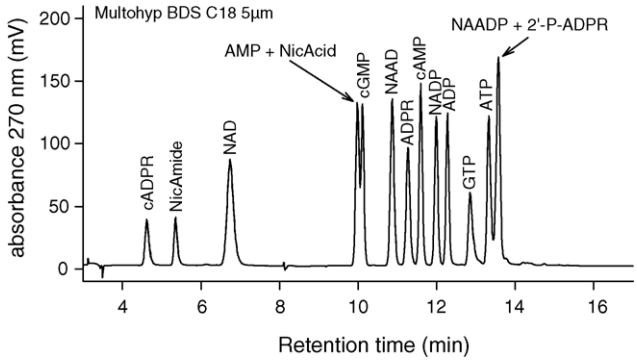

Fig. 1. Separation of ADPR by reversed-phase HPLC. A mixture of the indicated compounds (500 pmol–1 nmol each) was analyzed using a Multohyp BDS C18 column (250 mm × 4.6 mm, particle size 5 μm) at a flow rate of 1.0 ml/min with a gradient from buffer to methanol (see Section 2); abbreviations: cADPR, cyclic ADP-ribose, NicAmide, nicotinamide; NicAcid, nicotinic acid; NAAD, nicotinic acid adenine dinucleotide; NAADP, nicotinic acid adenine dinucleotide phosphate; 2′-P-ADPR, 2′-phospho adenosine diphosphoribose.

Experiments using artificial samples instead of cells were performed to investigate a possible degradation of NAD to ADPR, since NAD typically is present within cells in the high micromolar range [19]. This process might occur during the sample extraction, which is done in the presence of TCA. To analyze this potential degradation, artificial samples consisting of bovine serum albumin (BSA), and defined amounts of either NAD, ADPR, or both NAD and ADPR were prepared. These samples were then extracted using TCA and pre-purified by solid phase extraction in the same way as the samples consisting of cells. Upon analysis of the artificial samples containing BSA and NAD, no peak corresponding to ADPR was detected (Fig. 3B). Thus, NAD was completely separated during the solid phase extraction procedure. The artificial samples containing only ADPR showed an ADPR peak as expected (Fig. 3C). Importantly, no significant increase of this ADPR peak was found in the samples

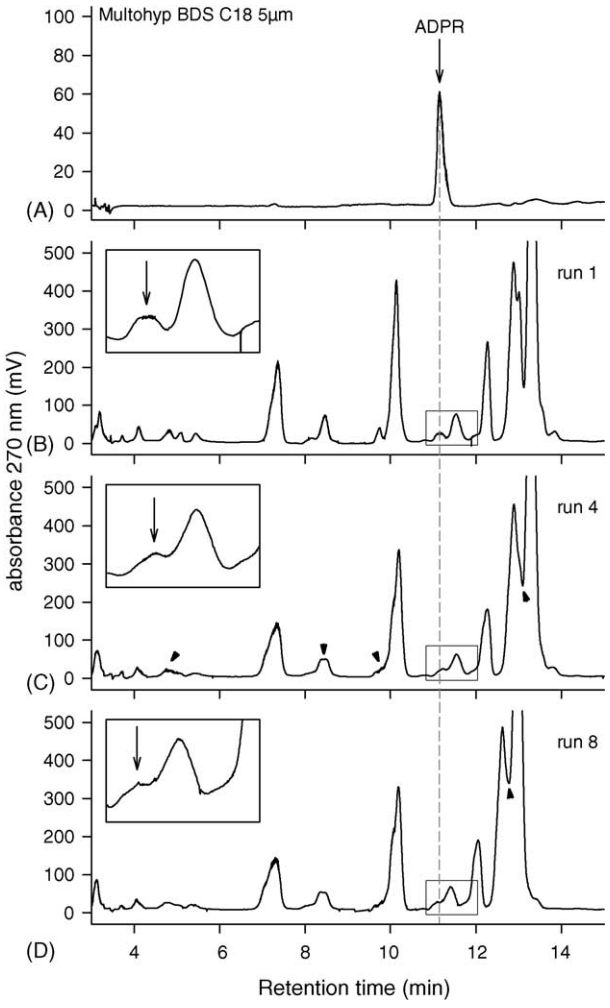

Fig. 2. Decrease of HPLC performance upon separation of crude nucleotide extracts. (A) One nanomole standard ADPR was analyzed by reversed-phase HPLC as described in the legend to Fig. 1. (B–D) 5×10^7 Jurkat cells were extracted with 20% (w/v) trichloroacetic acid, the extracts neutralized by extraction with diethyl ether, and aliquots corresponding to 5×10^6 cells were repeatedly separated by reversed-phase HPLC as above. First, fourth and eighth separation on the same column are depicted in (B–D), respectively. Insets show the ADPR peaks (arrows). Arrowheads indicate decrease of separation performance upon repeated analysis.

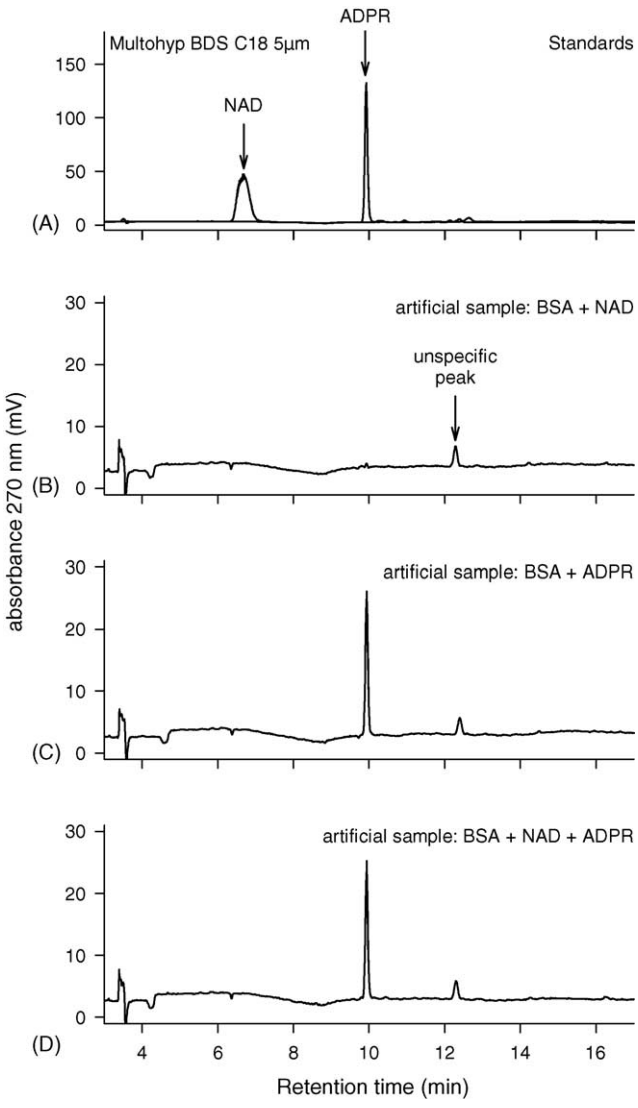

Fig. 3. Acidic extraction of nucleotides does not hydrolyze NAD into ADPR. (A) A mixture of 1 nmol NAD and 1 nmol ADPR was separated by reversed-phase HPLC as described in the legend to Fig. 1. (B–D) To exclude a significant degradation of endogenous NAD to free ADPR during the acidic extraction procedure, artificial samples consisting of BSA, NAD and/or ADPR as indicated were extracted and purified by solid phase extraction as detailed in the legend to Fig. 4. The eluates were then analyzed by reversed-phase HPLC using a C18 column. The NAD is completely removed during the solid phase extraction and thus cannot be detected in the eluates. Note that the amount of ADPR in the sample containing both NAD and ADPR (D) is not increased as compared to the sample containing only ADPR but no NAD (C), ruling out a degradation of NAD to ADPR. The chromatograms are representative for three independent experiments. The slight change in the retention times of the compounds as compared to Fig. 1 may be caused by the use of a new HPLC column.

containing both ADPR and NAD (Fig. 3D), ruling out a significant degradation of NAD to ADPR during the acidic extraction procedure.

Analysis of both halves of such pre-purified twin-samples using the Multohyp BDS C18 column revealed one single peak that increased when ADPR had been added at the beginning of the extraction procedure (Fig. 4B versus Fig. 4A). This peak co-eluted with ADPR standards (arrows in Fig. 4), suggesting it was genuine ADPR. Further proof of identity and purity of this peak are almost identical UV absorption spectra between 210 nm and 340 nm of the sample peak and the genuine ADPR peak (Fig. 4C). The differences between the peak areas of the twin-samples were used to calculate the recovery of the extraction procedure for each twin-sample, which amounted to $92 \pm 15\%$ ($n = 26$, mean $\pm$ S.D.).

Endogenous ADPR in Jurkat cells was determined from the half of each twin-sample without spike after correction for the respective recovery, and normalized to the cell volume (1.588 ± 0.136 µl per 10^6 cells, mean $\pm$ S.D., range 1.363–1.871 µl per 10^6 cells, $n = 26$) to obtain cytosolic concentrations. The mean intracellular ADPR concentration in Jurkat cells was determined to be 44 ± 11 µM ($n = 26$,

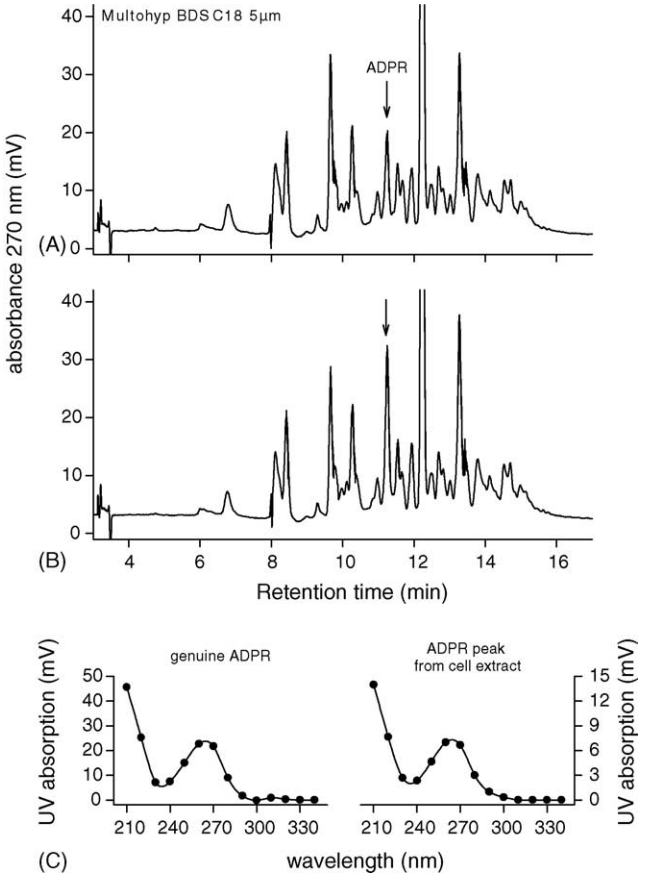

Fig. 4. HPLC analysis of endogenous ADPR in Jurkat T-lymphocytes using a C18 column. 5×10^7 Jurkat T cells were lysed by freeze–thaw-cycles in the presence of 20% (w/v) trichloroacetic acid, and the extracts divided into two identical halves at the beginning of the extraction procedure. After addition of 2.5 nmol ADPR to one half, the samples were purified by solid phase extraction using Q-sepharose, and eluted with 20 mM TFA. The extracts of 2×10^6 cells, either with (B) or without spike (A), were analyzed by reversed-phase HPLC as described in the legend to Fig. 1. The chromatograms are representative of 26 independent separations. (C) UV spectra of genuine ADPR (left panel) or ADPR peaks from cell extracts (right panel) were recorded at the timepoint marked by the arrow in (A) between 210 nm and 340 nm and corrected for background absorption (mean of $n = 3$).

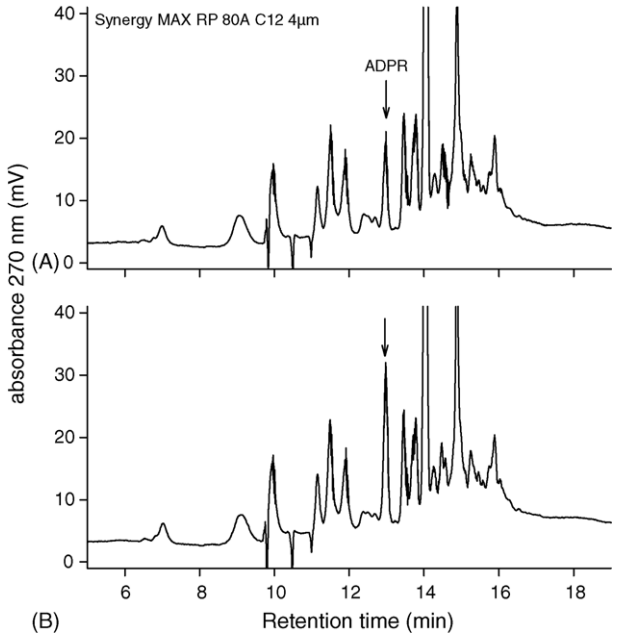

Fig. 5. HPLC analysis of endogenous ADPR in Jurkat T-lymphocytes using a C12 column. Extraction and purification of nucleotides from Jurkat T cells was done as described in the legend to Fig. 4. The extracts of 2×10^6 cells, either with (B) or without (A) ADPR spike, were analyzed by reversed-phase HPLC using a Syneryi MAX RP 80A C12 column (250 mm × 4.6 mm, particle size 4 µm) at a flow rate of 1.0 ml/min with a gradient from buffer to methanol (see Section 2). The chromatograms are representative of four independent separations.

mean ± S.D.), assuming an even distribution of endogenous ADPR within the cells and neglecting any solid volume inside the cells.

Intracellular ADPR was also measured in the murine thymoma cell line BW5147 using the Multohyp BDS C18 column (data not shown). Here, a cytosolic ADPR concentration of 73 ± 11 µM ($n = 5$, mean ± S.D.) was calculated, using a mean cellular volume of 0.754 ± 0.028 µl per 10^6 cells (mean ± S.D., range 0.697–0.768 µl per 10^6 cells, $n = 5$); in these experiments, the recovery was 68 ± 13% ($n = 5$, mean ± S.D.).

The results obtained using the C18 column were additionally checked using a second HPLC system. For this approach a reversed-phase column with a different chromatographic behaviour, a C12 surface (Synergi MAX RP 80A) was used and the samples were analysed in the same way as described for the C18 column. Using this column, the recoveries and the calculated ADPR concentrations in Jurkat T cells were similar to the results obtained with the C18 column (Fig. 5).

4. Discussion

In this contribution we established a HPLC based method for the quantification of intracellular levels of the putative messenger ADPR, which is supposed to regulate the activity of ion channels involved in Ca^{2+} influx into cells. After optimization of the extraction procedure, the cellular concentrations of ADPR were determined to be 44 ± 11 µM in the human T cell line Jurkat and 73 ± 11 µM in murine BW5147 thymoma cells. A degradation of NAD to free ADPR during the extraction was ruled out using artificial samples. Two types of reversed-phase columns differing in their surface properties (C18 versus C12) as well as the UV fingerprint of ADPR were used to demonstrate the purity of the peak that was identified as genuine ADPR. Taken together, these data for the first time constitute exact quantification of intracellular ADPR in two lymphatic cell lines.

4.1. Determination of ADPR

In order to accurately quantify ADPR, it is very important to immediately destroy metabolic enzymes present in

the cellular extracts. Cell lysis in the presence of TCA or perchloric acid (PCA) is the method of choice for the determination of intracellular nucleotides [9]. Protocols using PCA were tested for ADPR extraction, but resulted in higher variance of recoveries, possibly due to loss of nucleotides by co-precipitation with potassium perchlorate upon neutralization with KOH. Thus, lysis with TCA followed by ether extraction was chosen for further experiments. Since the direct analysis of crude neutralized TCA extracts was not possible due to contaminants causing rapid clogging of HPLC columns and decrease of separation performance, a solid phase extraction step was introduced in order to pre-purify the extracts. For the determination of intracellular concentrations of the Ca^{2+} mobilizing second messenger cADPR [15], we established a two-step HPLC method using a strong anion exchange (SAX) column followed by a reversed-phase column with a sensitivity of 10 pmol [15]. To quantify endogenous ADPR this scheme was adopted, but simplified by replacing the SAX-HPLC column with a solid phase extraction step using a SAX matrix with a step elution protocol. Although solid phase extraction usually results in less efficient pre-purification as compared to a HPLC step, this proved sufficient for ADPR. Also, any loss of ADPR by unspecific adhesion to plastic column walls, a crucial point in trace analysis of biological nucleotides occurring in small amounts, like cADPR [15] or nicotinic acid adenine dinucleotide phosphate (NAADP; Gasser and Guse, unpublished data) was not critical in case of ADPR due to its expected higher endogenous concentration. The latter was concluded since biological effects of cADPR occurred at around 10 μM [16,20], whereas concentrations between 100 μM and 300 μM were required for gating of TRPM2 [3–5]. The analytical data obtained, cytosolic concentrations of about 44 μM ADPR (this contribution) versus about 0.5 μM cADPR in Jurkat cells [16], finally confirmed our assumption.

Our HPLC assay showed a detection limit of 5 pmol ADPR, and a linear range up to 50 nmol combined with stable retention times (interday precision more than 99%), using a reversed-phase C18 column. Since the ADPR amounts are at least 50 pmol in 10^6 cells, this method is well suited to accurately quantify endogenous ADPR. Enzymatic or chemical treatments, like, e.g. pre-column fluorescence derivatization, were avoided to prevent potential systematic errors. The recovery of the whole sample preparation was between $68 \pm 13\%$ and $92 \pm 15\%$, a comparable value to other extraction methods for intracellular nucleotides [10,15]. Importantly, the eluates of the solid phase extraction were stored in glass vials, since the use of polypropylene tubes resulted in partial degradation of samples and/or release of contaminants from the tube-wall material leading to low resolution of the chromatograms. Additionally, HPLC analysis was done as soon as possible after preparation of the eluates to avoid problems related to degradation or release of unknown contaminants from tubes.

Since higher levels of free NAD [19] as compared to ADPR are present in cells, it is important to exclude a degradation of NAD under the acidic conditions necessary to denature cellular compounds. This degradation might lead to generation of free ADPR by cleavage of the *N*-glycosidic linkage between nicotinamide and ADPR in the NAD molecule. However, a significant degradation of NAD to ADPR during the sample preparation was ruled out using artificial samples consisting of BSA (instead of cellular protein) and either NAD, ADPR, or both NAD and ADPR.

For human erythrocytes, a method for the determination of intracellular ADPR has been described [20], consisting of three sequential fractionations of red blood cell extracts. However, because of much lower ADPR concentrations in erythrocytes and quite unusual conditions due to the hemoglobin present in these cells, this procedure seems hardly applicable to other cell types. Extracellular ADPR was quantified in the superfusates of mesenteric arteries after electric field stimulation [21]. For this purpose all nucleotides containing adenine moieties in the bath solution were derivatized by chloroacetaldehyde, resulting in the formation of fluorescent 1,N^6-etheno derivatives [11], which were analyzed by HPLC using fluorescence detection. Although low detection limits (10 fmol for 1,N^6-etheno ADPR) were reported [21], this method is not applicable for the analysis of total cellular nucleotide extracts because of the complexity of these samples (Gasser and Guse, unpublished data); in contrast to cellular extracts, the superfusates of mesenteric arteries contained only a very limited number of compounds [21].

For the quantification of related intracellular nucleotides two different approaches have been used: either direct quantification using HPLC or, more indirectly, enzymatic cycling assays, which take advantage of an amplification by two coupled enzymatic reactions that result in the generation of a fluorescent dye. HPLC based methods employing UV detection normally show detection limits in the low pmol range [10,15], or in the low fmol range for fluorescence detection [10,12,21]. Enzymatic cycling assays have been long known for molecules like NAD [13], NADP [14], or cGMP [22]. Recently, this type of reaction was also adopted for the quantification of the Ca^{2+} mobilizing compounds cADPR [23] and NAADP [24] with detection limits of 100–300 fmol. However, none of these methods can be easily adopted for the quantification of ADPR, since an enzymatic conversion of ADPR to NAD, which can be easily measured using enzymatic cycling reactions [13], is not possible. Also the major advantage of enzymatic cycling assays, i.e. the high sensitivity, is not required here, since our HPLC assay is well suited to quantify cellular ADPR levels, using a reasonable number of cells per sample.

4.2. ADPR as an endogenous modulator of cation channels?

In coronary arterial smooth muscle cells, ADPR concentrations of >0.1 μM dose-dependently increase the open probability of K_{Ca} channels [1,2]. Since the intracellular

ADPR concentrations determined in this contribution are far above this concentration, ADPR may act as a constitutive activator of K_{Ca} channels by constantly increasing their open probability, provided that arterial smooth muscle cells contain a similar intracellular ADPR concentration as compared to lymphocytes. In contrast to this, much higher ADPR concentrations (more than 60 μM) are necessary to induce currents of the non-selective cation channel TRPM2, with a half-maximal response at 90 μM [3–5], indicating that TRPM2 is not activated in quiescent T cells. Importantly, TRPM2 is co-activated by Ca^{2+} itself [25], leading to a feed-forward amplification by ADPR-induced Ca^{2+} influx, rise in local Ca^{2+} concentration, and subsequent co-activation of TRPM2. Activation of TRPM2 currents was induced by extracellular oxidative stress [6,26], or stimulation of cells with TNF-α [26], demonstrating an important role of the ADPR/TRPM2 system in cellular signaling. Since the basal cellular ADPR concentrations in T cells are either below or in the lower part of the range described for activation of TRPM2, most of the channels can be considered to be mainly in the closed state. However, a basal ADPR concentration close to a modulatory one opens up the possibility to quickly activate more of the channels by increasing the cellular ADPR amount, like it would be necessary for fast signaling processes, e.g. in apoptosis or reactions to oxidative stress. It is also important to note that the local ADPR concentrations in cells may somewhat differ from the mean values that were calculated for the whole cell volume. For the latter, the volume of the cells was calculated from their diameters, which does not take into account any solid structures reducing the liquid phase volume in the cells.

5. Conclusion

Since the ADPR-gated Ca^{2+} channel TRPM2 is widely expressed in cells of the immune system, e.g. monocytes and T-lymphocytes [3] and activation of leukocytes by inflammatory stimuli elicits Ca^{2+} influx as one major mechanism [27], it would be of great benefit to pharmacologically modulate this process. To reach this aim, the mechanisms mediated by diverse second messengers jointly generating intracellular Ca^{2+} signals must be understood in detail. Quantification of endogenous ADPR, one important step along this road, is now made possible using the HPLC method described in this contribution.

Acknowledgements

This study was partially supported by the Deutsche Forschungsgemeinschaft, the Wellcome Trust, and the Gemeinnützige Hertie-Stiftung. This article is based in part on a doctoral study by A.G. in the faculty of Chemistry, University of Hamburg.

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