A novel cycling assay for cellular cADP-ribose with nanomolar sensitivity

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cADP-ribose (cADPR) is a novel cyclic nucleotide derived from NAD^+ that has now been established as a general Ca^{2+} messenger in a wide variety of cells. Despite the obvious importance of monitoring its cellular levels under various physiological conditions, its measurement has been technically difficult and requires specialized reagents. In this study a widely applicable high-sensitivity assay for cADPR is described. ADP-ribosyl cyclase normally catalyses the synthesis of cADPR from NAD^+, but the reaction can be reversed in the presence of high concentrations of nicotinamide, producing NAD^+ from cADPR stoichiometrically. The resultant NAD^+ can then be coupled to a cycling assay involving alcohol dehydrogenase and diaphorase. Each time NAD^+ cycles through these coupled reactions, a molecule of highly fluorescent resorufin is generated. The reaction can be conducted for hours, resulting in more than a thousand-fold amplification of cADPR. Concentrations of cADPR in the nanomolar range can be measured routinely. The unique ability of ADP-ribosyl cyclase to catalyse the reverse reaction provides the required specificity. Using this assay, it is demonstrated that cADPR is present in all tissues tested and that the levels measured are directly comparable with those obtained using a radioimmunoassay. All the necessary reagents are widely available and the assay can be performed using a multiwell fluorescence plate reader, providing a high-throughput method for monitoring cADPR levels. This assay should be valuable in elucidating the messenger role of cADPR in cells.

Key words: ADP-ribosyl cyclase, Ca^{2+} signalling, CD38, NAD^+, NADH.

INTRODUCTION

The importance of cADP-ribose (cADPR) in mediating Ca^{2+} release from internal stores has been well established for a variety of systems, from protist and plant to human (reviewed in [1,2]). Pharmacological evidence and studies on cADPR-sensitive channels reconstituted into lipid bilayers indicate that the action of cADPR is to modulate the Ca^{2+} sensitivity of ryanodine receptor channels [3–10]. Additionally, cADPR has been proposed to be a Ca^{2+} messenger [11,12]. A necessary characteristic of a messenger is that its cellular levels are responsive to specific stimuli. The first demonstration that cADPR is present endogenously in tissues used a bioassay based on its Ca^{2+}-releasing activity [13,14]. This bioassay is highly specific, but requires the preparation of microsomes from sea urchin eggs, and its sensitivity is only moderate. Meticulous attention is also needed in eliminating Ca^{2+} contamination during sample preparation. Nevertheless, using this bioassay, it was shown that stimuli can indeed elevate cellular cADPR levels in plants as well as in invertebrate and mammalian cells [15–20]. A two-step HPLC method has also been developed for measuring endogenous cADPR levels in T-lymphocytes [21]. A more sensitive radioimmunoassay (RIA) has since been developed [22,23] and has been used to show that cADPR is present even in *Euglena*, a protist, and that its levels exhibit periodic variations that correlate with stages of the cell cycle [24]. To take advantage of the increased sensitivity of the RIA compared with previous assays, freshly radiolabelled cADPR with high specific activity is needed. Also, the necessary specific antibody against cADPR has not been generally available. These limitations have so far prevented the widespread application of these assays for cADPR, hampering advances in our understanding of the regulation of the cADPR-mediated signalling process. It was the purpose of this study to circumvent these limitations by developing an assay for cADPR with sensitivity comparable with the RIA, but employing readily available reagents.

MATERIALS AND METHODS

Materials

ADP-ribosyl cyclase was prepared by a yeast expression system, as described previously, and purified by cation-exchange chromatography [25]. Native cyclase isolated from *Aplysia* as described previously [26] is also available commercially. Alcohol dehydrogenase from yeast (suitable for cycling assays), activated charcoal, diaphorase, resazurin, Tris, NAD^+, nicotinamide, nucleotide pyrophosphatase from *Crotalus atrox* venom, NADase from *Neurospora crassa*, tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane were obtained from Sigma. Alkaline phosphatase from calf intestine was from Boehringer. Centricon-3 filters were from Millipore.

Cycling assay for cADPR

Typically, reactions were conducted in 96-well plates. To 0.1 ml of cADPR or other nucleotide samples, 50 μl of reagent was added containing 0.3 μg/ml ADP-ribosyl cyclase, 30 mM nicotinamide and 100 mM sodium phosphate, pH 8. This initiated the conversion of cADPR in the samples to NAD^+. The conversion was allowed to proceed for 15 min at room temperature ($\approx$ 25 °C). The cycling reagent (0.1 ml) was then added, which contained 2 % ethanol, 100 μg/ml alcohol dehydrogenase, 20 μM resazurin, 10 μg/ml diaphorase, 10 μM FMN, 10 mM nicotinamide, 0.1 mg/ml BSA and 100 mM sodium phosphate, pH 8. The cycling reaction was allowed to proceed for 2–4 h and the increase in the resorufin fluorescence (with excitation at 544 nm and emission at 590 nm) was measured periodically using

Abbreviations used: cADPR, cADP-ribose; NAADP, nicotinic acid adenine dinucleotide phosphate; NHD, nicotinamide hypoxanthine dinucleotide; RIA, radioimmunoassay.

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a fluorescence plate reader. The results shown are means ± S.D. from at least three independent measurements.

The commercial diaphorase was treated with charcoal to remove bound NAD^+. Typically, the lyophilized powder was diluted to 12 mg of protein/ml with water. Just prior to preparing the cycling reagent, 20 μl of enzyme solution was diluted with 60 μl of a 2 % charcoal suspension in 20 mM phosphate, pH 7, and the enzyme/charcoal suspension incubated for 30 min at 37 °C. The charcoal was removed by centrifugation for 5 min in a microfuge and the enzyme recovered from the supernatant. Alcohol dehydrogenase was purchased as suitable for enzyme cycling, and it was used without charcoal treatment.

Tissue and cell extracts

Rat tissues were frozen in liquid N_2, pulverized into a powder and extracted with 0.6 M perchloric acid at 4 °C. Perchloric acid was removed by mixing the aqueous sample with a solution containing 3 vol. of 1,1,2-trichlorotrifluoroethane to 1 vol. of tri-*n*-octylamine [27]. Then, 1 part sample was added to 4 parts organic phase and vortexed for 1 min. Following a centrifugation step for 10 min at 1500 ***g***, the tubes were maintained on ice until the aqueous phase easily separated from the organic phase. This aqueous layer containing the cADPR was removed and was adjusted to pH 8 with 20 mM sodium phosphate.

To remove all contaminating nucleotides, a mixture containing hydrolytic enzymes was added to the samples with the following final concentrations: 0.44 unit/ml nucleotide pyrophosphatase, 12.5 units/ml alkaline phosphatase, 0.0625 unit/ml NADase, 2.5 mM $MgCl_2$ and 20 mM sodium phosphate, pH 8.0. The incubation proceeded overnight at 37 °C. The enzyme mixture hydrolysed all nucleotides, including NAD^+, in the samples, except cADPR, which is resistant to these enzymes [23]. Enzymes were removed by filtration with Centricon-3 filters and samples were recovered in the filtrate after centrifugation at 4 °C and 3000 ***g*** for 30 min using a Beckman JA-20 rotor. RIAs were conducted exactly as described previously [23].

Endogenous NAD^+ in perchloric acid extracts of rat tissues can also be measured. Following neutralization, NAD^+ in the samples was converted into NADH in the presence of 1 % ethanol and 9 μg/ml alcohol dehydrogenase in 100 mM Tris/HCl, pH 8. The resultant increase in NADH fluorescence was measured in a multiwell plate reader (with excitation at 355 nm and emission at 460 nm). Alternatively, the acid extract was diluted 400-fold in 100 mM sodium phosphate buffer, pH 8, and assayed by the complete cycling assay as described for cADPR. The recovery of NAD^+ and cADPR was 80 % after extraction with 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine. Standard solutions of cADPR were prepared in 20 mM sodium phosphate, pH 8.0, and taken through the same steps as the samples.

HL60 cells were seeded at an initial density of 0.2×10^6 cells/ml and maintained in RPMI 1640 medium containing 10 % fetal bovine serum. The cells were induced to differentiate with 1 μM retinoic acid. After 3 days, $\approx 6 \times 10^6$ cells were pelleted by centrifugation and extracts were prepared by the addition of 0.5 ml of 0.6 M perchloric acid.

RESULTS

Conversion of cADPR into NAD^+

ADP-ribosyl cyclase is a novel multi-functional enzyme. It catalyses the cyclization of NAD^+ to produce cADPR and nicotinamide [26]. The cyclase can also use NADP as a substrate and, in the presence of nicotinic acid, catalyses a transglycosylation reaction that exchanges the nicotinamide group of NADP with nicotinic acid, producing nicotinic acid adenine dinucleotide phosphate (NAADP) [28,29]. NAADP has also been shown to be a Ca^{2+} messenger in a wide variety of cells and mediates the mobilization of intracellular Ca^{2+} stores that are separate from those sensitive to cADPR ([30], reviewed in [2,31]). Structure–function studies using a series of analogues of nicotinic acid show that nicotinamide is equally effective in supporting the base-exchange reaction [32]. This raises the possibility that the cyclization of NAD^+ catalysed by the cyclase may be reversible [33]. This is indeed the case, as shown in Figure 1. Incubation of cADPR with nicotinamide and the cyclase resulted in a time-dependent conversion into NAD^+, as assayed by HPLC (Figure 1A). The reaction did not go to completion but levelled off when about 80 % of the cADPR was converted. One way to shift the steady state towards complete conversion of cADPR is to remove the product, NAD^+. This was achieved by including alcohol dehydrogenase in the reaction mixture, which reduces NAD^+ to NADH in the presence of alcohol. As shown in Figure 1(B), under these conditions, cADPR was rapidly (within 2 min of incubation) and completely converted into NADH. Stoichiometric conversion of cADPR into NADH could be demonstrated over a range of cADPR concentrations, as shown in Figure 2. The sensitivity of detection by HPLC was in the low micromolar range, which is not sufficient for use in measuring endogenous cADPR in cells.

Amplification by an enzyme-coupled cycling assay

To increase the sensitivity of the assay, we made use of an enzymic cycling method that has long been used for detection of cGMP and NAD^+ [34,35]. The design of the assay is illustrated in Scheme 1. cADPR is first converted into NAD^+ using the cyclase in the presence of a high concentration of nicotinamide. The NAD^+ produced is then fed into the coupled-enzyme cycling reaction, consisting of alcohol dehydrogenase and diaphorase (Scheme 1). The former reduces NAD^+ to NADH, while the latter cycles NADH back to NAD^+ with the production of a highly fluorescent resorufin molecule from the non-fluorescent substrate, resazurin. The number of cycles can be controlled by the time of incubation.

Verification of the cycling assay

We first verified the cycling reactions with nanomolar concentrations of NAD^+. The amplification of NAD^+ resulted in a linear increase in resorufin fluorescence for up to 2 h (results not shown). The linear relation held for all concentrations of NAD^+ tested (0–100 nM). The rates of resorufin fluorescence increase were obtained from the slopes of the linear regression lines, and they were perfectly linear ($r^2 = 0.999$) with respect to NAD^+ concentration. The linearity of the assay is quite remarkable considering that two different enzyme reactions are coupled. The assay is thus clearly sensitive enough to detect 1–2 nM NAD^+ (results not shown). Typically, an incubation of 2–5 h amplified the starting NAD^+ more than a thousand times, producing micromolar concentrations of resazurin from nanomolar concentrations of NAD^+. This cycling reaction can be monitored conveniently in a fluorescence multiwell plate reader, which can be set to measure the increase in resazurin fluorescence periodically. It is thus a one-step assay, not requiring any further separation or manipulation of the samples, making it highly convenient and reproducible.

A similar concentration range of cADPR was tested next. Samples were first incubated with the cyclase in the presence of 10 mM nicotinamide and the cycling reaction mixture was then

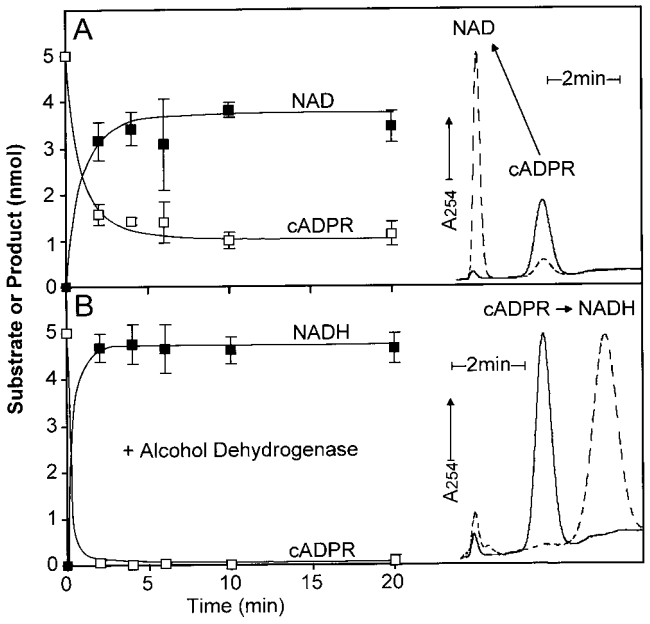


Figure 1 Reversal of the cyclization reaction catalysed by ADP-ribosyl cyclase

cADPR (5 μM) was incubated with the cyclase (0.1 μg/ml) in the presence of a high concentration of nicotinamide (10 mM) at pH 8. At the times indicated, samples were collected from the reaction mixture and analysed by HPLC. (**A**) The reversal of the cyclase reaction resulted in the time-dependent conversion of cADPR into NAD^+. The HPLC chromatographs of the reversal reaction at time 0 (right-hand panel, solid line) and at 20 min (right-hand panel, dashed line). (**B**) Inclusion of alcohol dehydrogenase (9 μg/ml) and ethanol (1 %) allowed the complete conversion of cADPR into NADH to occur within minutes.

added. Figure 3(A) shows that both the amounts of resorufin produced (resorufin fluorescence) and the linearity of the assay were very similar to those seen with NAD^+ standards, indicating that the conversion of cADPR into NAD^+ was stoichiometric. Figure 3(B) shows a linear relationship between the rate of resorufin fluorescence increase and starting cADPR concentration. As expected from the design of the assay, resorufin produced from cADPR absolutely required the presence of the cyclase (Figure 3B), which is in contrast to NAD^+ (Figure 3B, inset). The difference in resorufin fluorescence produced in the presence and absence of the cyclase thus provided a convenient means to distinguish between contributions of authentic signals from that of the background, such as contaminating NAD^+. Even at the lowest concentration of cADPR tested (0.5 nM), the rate of resorufin production was significantly different from the control without cADPR ($P < 10^{-5}$; Student's t test). The cycling assay thus has a sensitivity better than 0.5 nM cADPR. Since the assay volume was 0.1 ml, the sensitivity of the cyclase assay is better than 50 fmol of cADPR.

Resorufin production from cADPR should require not only the presence of the cyclase but also nicotinamide. This was found to be the case. The half-maximal concentration of nicotinamide was determined to be about 0.6 mM and was close to maximal at about 5 mM (results not shown). This dependence is due solely to the reversal of the cyclase reaction. Other enzymes in the cycling assay, such as alcohol dehydrogenase or diaphorase, are not affected by this concentration of nicotinamide when tested separately (results not shown).

The specificity of the cycling assay was tested with a series of nucleotides, as listed in Table 1. As expected, cADPR, NAD^+ and NADH all produced similar signals. None of the other nucleotides tested was effective, except nicotinamide hypoxanthine dinucleotide (NHD), which produced a small signal. This high degree of selectivity is clearly the result of the high specificity of the cyclase as well as the cycling enzymes, alcohol dehydrogenase and diaphorase.

If the cycling assay is to be applicable for measuring cADPR in cell extracts, the background signals from NAD^+ or NADH need to be eliminated. This was done by treatment with a combination of nucleotide-hydrolysing enzymes, including NADase, nucleotide pyrophosphatase and alkaline phosphatase (Table 2, +Enz). Table 2 shows that in the absence of the cyclase, cADPR did not produce any signal. The signal produced by cADPR in the presence of the cyclase was not affected by the enzyme treatment since cADPR was resistant to the hydrolytic enzymes [23]. However, cADPR was sensitive to CD38, which specifically hydrolyses cADPR to ADP-ribose [36] and completely eliminated the cADPR signal. In contrast to cADPR, signals produced by NAD^+, NADH and NHD did not require the presence of the cyclase and were completely eliminated by treatment with hydrolytic enzymes.

To further validate the cycling assay, the standard curves of cADPR were determined in the presence and absence of cell extracts. HL-60 cells were induced to differentiate with retinoic acid (1 μM), which resulted in expression of CD38 and production of cADPR [22,23]. After 3 days of treatment, cell extracts were prepared as described in the Materials and methods section. The cADPR standard curve remained linear ($r^2 = 0.997$; results not shown). The slope of the regression was slightly (10 %) less than that in the absence of the cell extracts. This is most probably due to a slight quenching of the resorufin fluorescence by the extracts. The y-intercept of the standard line gave a value of 1.16 ± 0.08 pmol of cADPR/10^6 cells, which compares well with the value of 1.5 pmol/10^6 cells determined previously by RIA [22].

Endogenous cADPR and NAD^+ levels in rat tissues

Rat tissue extracts were first treated with the enzyme mixture to remove endogenous NAD^+ and other interfering nucleotides. The cycling assay was then performed in the presence and absence of ADP-ribosyl cyclase. The latter condition allowed quantification of the background contribution by residual NAD^+ and other interfering nucleotides. The difference in the resorufin signal in the presence and absence of the cyclase was calibrated using cADPR standards. Figure 4(A) shows that cADPR was present in all tissues tested, in the range of 1–2.5 pmol/mg. cADPR levels were highest in brain, spleen, lung and the small intestine, which is composed of mostly smooth muscle. Endogenous levels in the heart and liver were also measured using RIA (Figure 4A, white bars) and the results were essentially the same as those measured by the cyclase assay. As a further assurance of the authenticity of the resorufin signal, tissue extracts were treated with CD38 to hydrolyse the endogenous cADPR to ADP-ribose [23,36], which indeed eliminated completely the signals (results not shown).

As can be seen in the scheme of the cycling assay (Scheme 1), endogenous NAD^+ levels can be measured by conversion into NADH. Tissue extracts were incubated with or without alcohol dehydrogenase and the difference in NADH fluorescence produced was calibrated with NADH standards. Figure 4(B) shows that the tissue NAD^+ levels were a thousand times higher than those of cADPR and were in the range of 1–3 nmol/mg, well within the detection limits of the fluorimetric measurement for NADH.

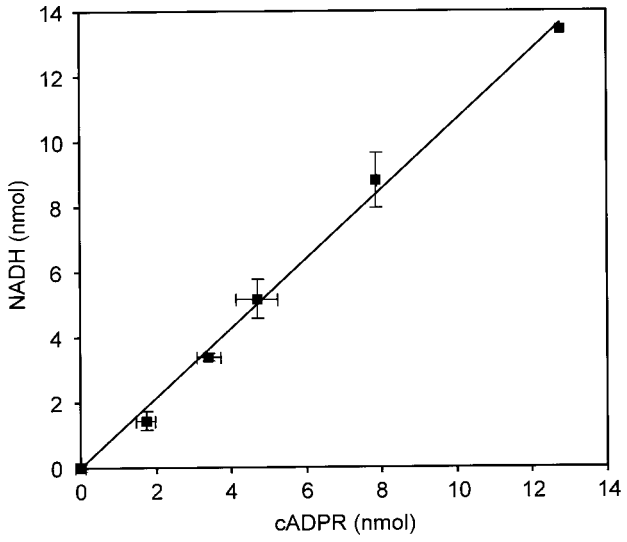
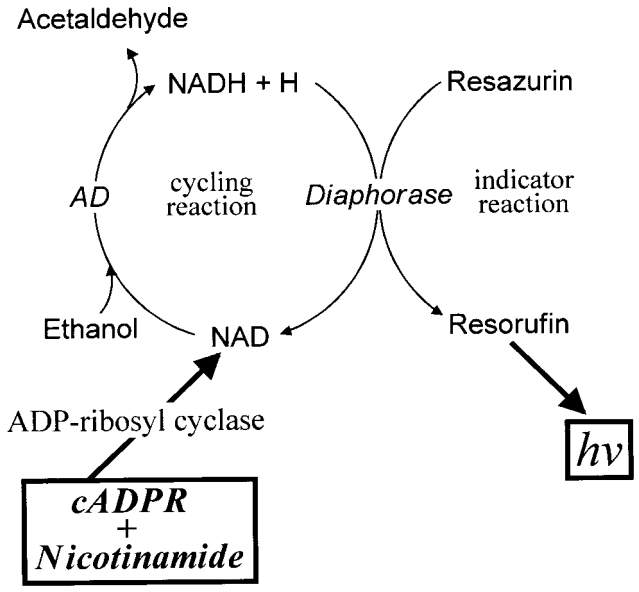


Figure 2 Stoichiometric conversion of cADPR into NAD$^+$ by ADP-ribosyl cyclase

The conditions were as described in Figure 1.



Scheme 1 The cycling assay for cADPR

Abbreviations used: AD, alcohol dehydrogenase; *hv*, fluorescence light.

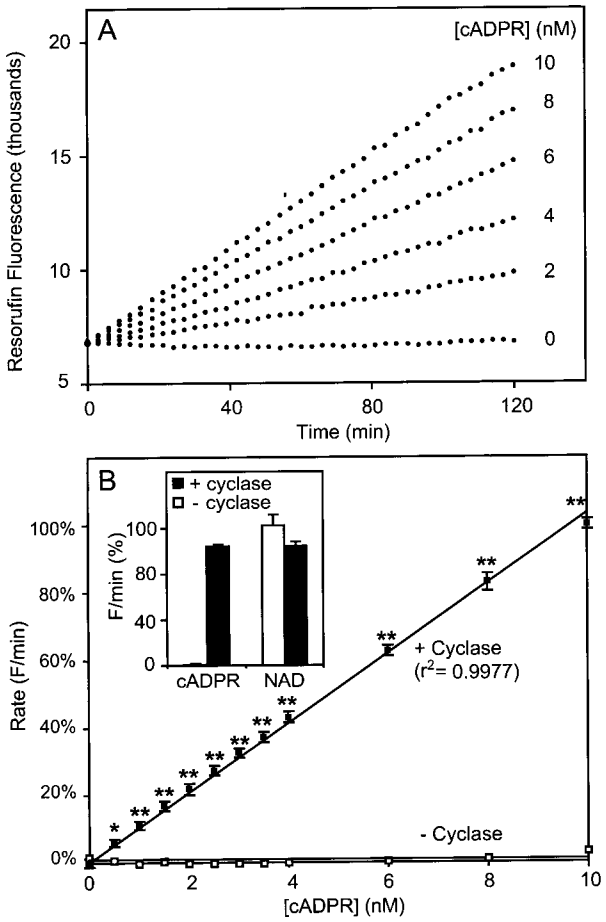


Figure 3 The cycling assay for cADPR

(**A**) Various concentrations of cADPR were incubated with the complete mixture of the cycling assay, including the ADP-ribosyl cyclase (+cyclase), as described in the Materials and methods section. The resultant continuous increase in resorufin fluorescence was measured periodically using a multiwell plate reader. (**B**) The rates of resorufin fluorescence increase were obtained by linear regression analyses of fluorescence time courses and plotted against cADPR concentration. In the absence of the cyclase (−cyclase), no increase in resorufin fluorescence was observed. The inset contrasts the cycling assay for cADPR and NAD$^+$. For cADPR the assay was completely dependent on the presence of the cyclase, whereas, in the case of NAD$^+$, it was totally independent of the cyclase. Values for all cADPR concentrations are significantly higher than in the absence of cADPR. $^*P < 10^{-5}$, $^{**}P < 10^{-7}$ (Student's *t* test).

DISCUSSION

Currently, three different assays for cADPR have been reported. The HPLC assay is the least sensitive, capable only of detecting 10 pmol of cAPDR [21]. The bioassay is slightly more sensitive, at $\approx$ 4 pmol [14]. The cycling assay for cADPR described in this study is about 100-fold more sensitive and is capable of detecting as low as 50 fmol of cADPR, which is comparable with the 100 fmol sensitivity of the RIA [22]. In addition to sensitivity, the cycling assay has two other major advantages over all currently available assays. First, all components of the assay, including the coupled enzymes, are available commercially, allowing this assay to be widely adopted. Secondly, the sensitivity of the assay is in the nanomolar range, which is comparable with the RIA, currently the most sensitive assay for cADPR. Indeed, the sensitivity of the cycling assay can be easily increased further to sub-nanomolar range by either prolonging the cycling reaction or reducing the reaction volume. Unlike the RIA, the cycling assay does not require the synthesis and purification of radioactive cADPR with high specific activity, nor does it require specific antibodies against cADPR. The remarkable linearity of the cycling assay (see Figure 2) also greatly improves the accuracy and simplifies the procedure for calibration. This is in contrast to the RIA, requiring the use of curvilinear binding curves. Moreover, it is a one-step fluorimetric assay that requires no further manipulation of the samples, making it highly reproducible.

Another noteworthy feature of the cycling assay is its high degree of specificity. Of the more than 17 related nucleotides tested, only NAD$^+$ and NADH, and to a much lesser degree,

Table 1 Specificity of the cycling assay

Experimental conditions were the same as described in the legend of Figure 3, except that 200 nM of various nucleotides were used. Results shown are means ± S.D. from four independent measurements. NAAD, nicotinic acid dinucleotide; NHD, nicotinamide hypoxanthine dinucleotide; NGD, nicotinamide guanine dinucleotide; ADPR, ADP-ribose; cGDPR, cGDP-ribose; cADPRP, cADPR phosphate.

	Rate of resorufin production (%)
NAD^+	100.0 ± 3.5
NADH	98.9 ± 2.4
cADPR	95.7 ± 0.8
NHD	7.5 ± 0.0
NGD	1.2 ± 0.4
NADP	0.4 ± 0.1
NAADP	0.3 ± 0.6
AMP	0.3 ± 0.3
NADPH	0.2 ± 0.2
cGDPR	0.1 ± 0.5
GTP	0.0 ± 0.1
cGMP	−0.1 ± 0.4
cADPRP	−0.1 ± 0.1
ADP	−0.2 ± 0.1
ATP	−0.2 ± 0.1
cAMP	−0.3 ± 0.2
NAAD	−0.3 ± 0.1
ADPR	−0.3 ± 0.1

NHD, are interfering. Potential interfering nucleotides can be removed readily by a mixture of hydrolytic enzymes, to which cADPR is remarkably resistant. cADPR can be, however, specifically hydrolysed by CD38, providing a diagnostic test for the assay. In this respect, the complete dependence of the assay on the presence of ADP-ribosyl cyclase (see Figure 4) provides an additional way in which to discriminate against artifact. These advantages and safeguards make the cycling assay the best method ever devised for measuring cADPR. More importantly, the remarkable simplicity of the assay readily lends itself to automation. The possibility of high-throughput screening of a wide variety of cells and tissues under various physiological conditions is a long-sought-after goal that will lead to eventual understanding of the regulation of this novel signalling pathway mediated by cADPR.

Table 2 Enzymic treatment of nucleotides

Experimental conditions were the same as in Table 1, except that the cycling reaction was done in the presence (+cyclase) or absence (−cyclase) of the cyclase. Treatment of NAD^+, NADH and NHD with a mixture of hydrolytic enzymes (+Enz) was carried out at 37 °C for 15 h as described in the Materials and methods section. Nucleotides were also incubated with 25 μg/ml CD38 (+Enz+CD38) for 15 h at 37 °C. Results shown are means ± S.D. from four independent measurements.

	Rate of resorufin production (%)	
Nucleotide and treatment	−Cyclase	+Cyclase
NHD	9.1 ± 0.2	3.3 ± 0.0
NHD + Enz	−0.7 ± 0.1	−0.7 ± 0.1
NHD + Enz + CD38	−0.6 ± 0.2	−0.8 ± 0.1
NAD^+	100.0 ± 1.3	93.3 ± 0.7
NAD^+ + Enz	−0.6 ± 0.1	−0.7 ± 0.0
NAD^+ + Enz + CD38	−0.7 ± 0.1	−0.7 ± 0.0
cADPR	−0.4 ± 0.1	90.7 ± 0.6
cADPR + Enz	−0.6 ± 0.2	90.7 ± 0.6
cADPR + Enz + CD38	−0.7 ± 0.1	−0.6 ± 0.1
NADH	89.4 ± 0.8	87.4 ± 1.3
NADH + Enz	−0.5 ± 0.1	−0.2 ± 0.2
NADH + Enz + CD38	−0.5 ± 0.2	−0.4 ± 0.2

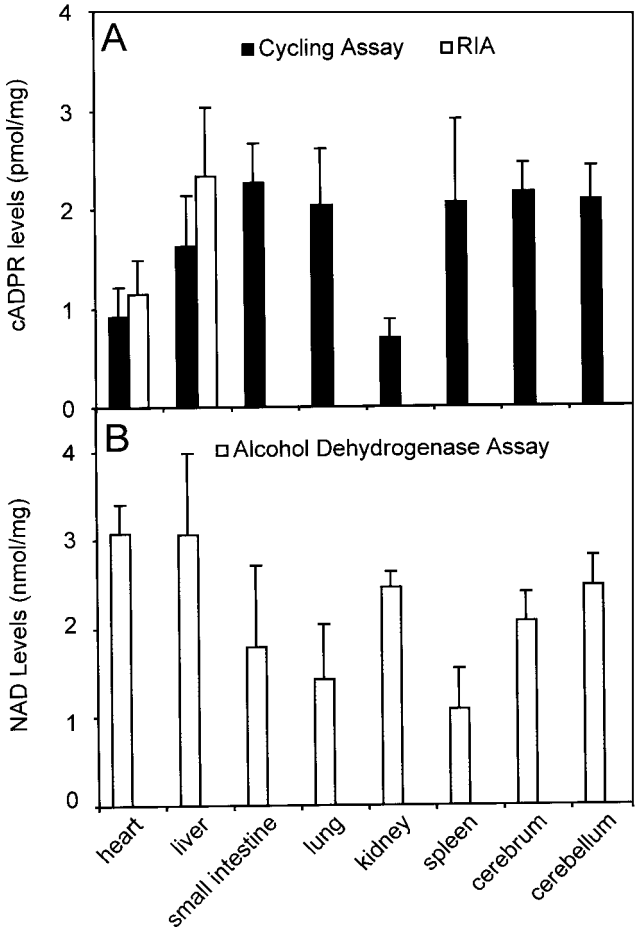


Figure 4 Endogenous cADPR and NAD^+ levels in rat tissues

(**A**) Tissues were extracted as described in the Materials and methods section. The cycling assay was done in the presence and absence of the cyclase. The differences in the resorufin signals were calibrated with cADPR standards taken through the entire extraction and incubation procedures. The endogenous levels of cADPR in the heart and liver were also measured using the RIA (white bars). The results were comparable with those obtained by the cycling assay (black bars). (**B**). Endogenous NAD^+ levels in tissues were measured using the alcohol dehydrogenase reaction as described in the Materials and methods section. Extracts were incubated with or without the dehydrogenase and the differences in NADH fluorescence signals were calibrated using NADH standards.

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