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Accumulation of cyclic ADP-ribose measured by a specific radioimmunoassay in differentiated human leukemic HL-60 cells with all-*trans*-retinoic acid

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Abstract Cyclic adenosine diphosphoribose (cADPR) is a novel candidate for the mediator of Ca^{2+} release from intracellular Ca^{2+} stores. The formation of this cyclic nucleotide is catalyzed by not only *Aplysia* ADP-ribosyl cyclase but also an ecto-form enzyme of NAD^+ glycohydrolase (NADase), which was previously identified as all-*trans*-retinoic acid (RA)-inducible CD38 in human leukemic HL-60 cells. In the present study, we developed a radioimmunoassay specific for cADPR, by which more than 100 fmol of cADPR could be detected without any interference by other nucleotides. The possible involvement of CD38 in the formation of cellular cADPR was investigated with the radioimmunoassay method. A marked increase in cellular cADPR was accompanied by all-*trans*-RA-induced differentiation of HL-60 cells. Moreover, a high level of cellular cADPR was observed in other leukemic cell lines, in which CD38 mRNA was expressed. Thus, CD38, which was initially identified as an NADase, appeared to be responsible for the formation of cellular cADPR.

Key words: ADP-ribosyl cyclase; CD38 antigen; cyclic ADP-ribose; NAD^+ glycohydrolase

1. Introduction

Cyclic adenosine diphosphoribose (cADPR), a metabolite of β-NAD^+, was first discovered in sea urchin eggs by Lee and his colleagues as a novel intracellular messenger with Ca^{2+}-mobilizing activity [1]. It is expected that the cyclic nucleotide mediates Ca^{2+} release from an inositol 1,4,5-triphosphate-insensitive Ca^{2+} store in sea urchin eggs [2–4]. Such Ca^{2+}-mobilizing activity of cADPR has also been observed in permeabilized rat pituitary cells [5], rat pancreatic islet β-cells [6], dorsal root ganglion cells [7], and a rat brain microsome fraction [8,9]. An increase in the cellular cADPR content was suggested in fertilizing sea urchin eggs [10] and glucose-stimulated pancreatic islet cells [6] by bioassays of a Ca^{2+}-releasing activity and/or the measurement of its amount on column chromatography [11–13]. However, these assays are not sufficient for precise determination of the cellular cADPR content in terms of specificity and sensitivity.

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Abbreviations: cADPR, cyclic adenosine diphosphoribose or cyclic ADP-ribose; IC_{50}, half-maximum inhibitory concentration; NMN^+, nicotinamide mononucleotide; PCR, polymerase chain reaction; RA, all-*trans*-retinoic acid; RIA, radioimmunoassay; NADase, NAD^+ glycohydrolase.

The cADPR-synthesizing enzyme, termed ADP-ribosyl cyclase, is especially abundant in *Aplysia* ovotestis, from which it was purified as a 29-kDa cytoplasmic protein and sequenced [14–16]. Interestingly, the ADP-ribosyl cyclase has an amino acid sequence homologous to that of CD38 antigen [17]. CD38 is a 46-kDa single-transmembrane glycoprotein with a short N-terminal cytoplasmic domain and a long C-terminal extracellular domain [18], and its expression is widely used as a phenotypic marker of the differentiation or activation of T and B lymphocytes [18–21]. We previously reported that ecto-form activity of NADase induced by all-*trans*-RA in HL-60 cells was due to the extracellular domain of CD38 [22]. Moreover, it has been reported that CD38 catalyzes not only the hydrolysis of β-NAD^+, but also the formation and hydrolysis of cyclic ADP-ribose to a lesser extent [23–27]. Thus, the two enzymes, which utilize β-NAD^+ as a substrate and cleave its *N*-glycoside bond, appear to be structurally and functionally related to each other.

In the present study, we first developed a RIA for the measurement of the cellular cADPR content. The present RIA method, which exhibits reasonable specificity for and sensitivity to cADPR with prior treatment of the sample with enzymes, was applied to studies on the possible involvement of CD38 in the formation of cellular cADPR.

2. Materials and methods

2.1. Purification of ADP-ribosyl cyclase from Aplysia ovotestis and preparation of cADPR

ADP-ribosyl cyclase was purified from ovotestis of *Aplysia kurodai*, a species common around the Japanese coast, as described previously [15,27]. cADPR was generated by incubation of the enzyme with β-NAD^+ and purified on an AG MP-1 column (Bio-Rad) as its free-acid form [27]. The free-acid form of cADPR (more than 98% purity) was converted to the sodium salt by means of chromatography on the sodium form of Dowex 50W X 8 (Bio-Rad), and then stored at −80°C. The structure of cADPR thus obtained was confirmed by NMR analysis [1,28], and its biological activity was supported by an ability to release Ca^{2+} from the microsomal fractions of sea urchin eggs [29]. [^{32}P]cADPR was also enzymatically prepared from [^{32}P]NAD^+ as follows. Eighteen μl of 6 μM [α-^{32}P]NAD^+ (29.6 TBq/mmol; NEN) was mixed with 3 μl of the purified ADP-ribosyl cyclase (200 μg/ml) in 0.13 M HEPES-NaOH (pH 7.4), and then the mixture was incubated at 25°C for 4 min. The reaction was terminated by adding 2 μl of 50% (w/v) trichloroacetic acid (TCA). After the addition of 5 μl of a 2 M Tris-base solution to adjust the pH to 6–7, the sample was diluted with 270 μl of water and then applied to the AG MP-1 column (0.4-ml bed volume) equilibrated with water. The column was washed with 1 ml of water, and bound [^{32}P]cADPR was eluted with 30 mM trifluoroacetic acid. Approximately 150 μl of 250–300 nM [^{32}P]cADPR was obtained, with recovery being more than 40%.

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2.2. Preparation of anti-cADPR antiserum

The purified cADPR was reacted with succinic anhydride to form the spacer-bound cADPR under the conditions reported previously [30], and the resulting succinyl cADPR was purified on the AG MP-1 column. The succinyl cADPR was then coupled with bovine serum albumin (BSA) to prepare the antigen as follows. Six mg of water-soluble carbodiimide was added 4 times at 1-h intervals to 2 ml of 0.1 M Na-phosphate (pH 6.4) containing 12 mg/ml of BSA and 2 mg/ml of succinyl cADPR. The pH was adjusted to 6–7 with HCl or NaOH during the incubation at 25°C. The reaction mixture was applied to a gel-filtration column (50-ml bed volume; Sephacryl S-100 HR), and the cADPR-BSA conjugate was eluted with phosphate-buffered saline (PBS). Approximately 2 mol of cADPR was incorporated per mol of BSA under the present conditions. The cADPR-BSA conjugate, as an antigen (approximately 1 mg of protein), was emulsified with Freund's complete adjuvant and injected into rabbits. Booster injection with 1 mg of the antigen were given five times at 2-week intervals. The immunized animals were bled by cutting a carotid artery 1 week after the last injection. The antiserum obtained was heated at 56°C for 30 min and stored at −80°C until use.

2.3. The standard procedure of RIA for cADPR

Crude preparations (cells or tissues) to be assayed for the cADPR content were mixed with a TCA solution at the final concentration of 4% and kept on ice for 20 min. After centrifugation at 15,000 × *g* for 10 min, the clear supernatant was neutralized with the 2 M Tris-base solution at a pH of approximately 7.5 (TCA extract). The TCA extract was first incubated at 25°C for 2 h with 50 U/ml of bovine alkaline phosphatase and 2 U/ml of venom phosphodiesterase in 100 mM imidazole-HCl (pH 7.5), 2 mM $MgCl_2$, 100 mM NaCl, and 400 mM KCl to degrade nucleotides that had cross-reacted with the anti-cADPR antiserum (see section 3). The enzyme-treated sample (or TCA extract) could be stored at −80°C for at least one month. For the standard RIA, 15 μl of a sample containing cADPR (4–512 nM) was mixed with 10 μl of 0.3–1.5 nM [^{32}P]cADPR (15,000–20,000 cpm) in 0.2 M imidazole-HCl (pH 7.5) containing 10 mg/ml of bovine γ-globulin, followed by the further addition of 5 μl of the anti-cADPR antiserum diluted with PBS containing 15 mg/ml of bovine γ-globulin. After incubation at 25°C for 2 h, 60 μl of 20% (w/v) polyethylene glycol (MW; 6,000) in 0.1 M imidazole-HCl (pH 7.5) was added to the reaction mixture. The immune complex of [^{32}P]cADPR-bound antibodies, together with γ-globulin added as a carrier, was precipitated by centrifugation at 15,000 × *g* for 10 min, and the supernatant (60 μl) was counted for the unbound form of [^{32}P]cADPR in a liquid-scintillation counter.

2.4. Separation of nucleotides by AG MP-1 column chromatography

A TCA extract prepared from cells was diluted with more than 9 volumes of water to reduce the salt concentration, and then applied to and eluted from the AG MP-1 column (0.5 × 10 cm) on a HPLC system. Nucleotides were eluted at the flow rate of 2 ml/min with the following gradient of 0 mM (0%) to 150 mM trifluoroacetic acid (100%) in water; 0% for 4 min, linear increase to 30% over 6 min, increase to 100% over 1 min, and then holding at 100% for 2 min. The absorbance at 254 nm was monitored. The retention times of standard nucleotides, NAD^+, AMP, cADPR, and ADP-ribose, were 5.3, 5.6, 7.3, and 9.8 min, respectively, under the present conditions.

2.5. Assay of NADase activity

Membrane fractions obtained from HL-60 cells were incubated at 30°C for 15–60 min with 200 μM β-NAD^+ in 50 μl of 20 mM Na-HEPES (pH 7.4), 1 mM $MgCl_2$, and 150 mM NaCl. The reaction was terminated by adding 2 μl of 100% (w/v) TCA, followed by standing on ice for 10 min. After centrifugation at 15,000 × *g* for 10 min, the clear supernatant was neutralized with the 2 M Tris-base solution, and then subjected to the AG MP-1 column as described previously [27].

2.6. Miscellaneous

HL-60 cells were cultured and caused to differentiate by various inducers as described previously [31–33]. MOLT-4 and THP-1 were also cultured in RPMI-1640 supplemented with 10% fetal bovine serum. All-*trans*-retinoic acid, and bovine alkaline phosphatase (P-7915, Type II-G) were obtained from Sigma (MO, USA). Venom phosphodiesterase I (*Crotalus adamanteus* venom) was purchased from Worthington Biochemical Co. (NJ, USA). Nicotinamide, succinic anhydride, water-soluble carbodiimide, and polyethylene glycol (MW; 6,000) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dibutyryl cAMP was from Yamasa Shouyu, Inc. (Tokyo, Japan). The other reagents used were of analytical grade. Protein concentrations were estimated with a DC Protein Assay Kit (Bio-Rad).

3. Results

3.1. Characterization of anti-cADPR antiserum and development of RIA for cADPR

We first investigated the properties of the anti-cADPR antiserum obtained in the present study for the development of a RIA for cADPR. When 0.1–0.5 nM [^{32}P]cADPR (15,000–20,000 cpm) was incubated at 25°C for 2 h with the antiserum at an appropriate dilution in a total volume of 30 μl, 60–70% of the added [^{32}P]cADPR bound to the antiserum. Fig. 1 shows the specificity of the anti-cADPR antiserum, which was determined from the displacement curves for various nucleotides. As expected, non-radiolabeled cADPR inhibited [^{32}P]cADPR binding to the antiserum, with an IC_{50} value of 20 nM. In addition, NAD^+ and NMN^+ inhibited the [^{32}P]cADPR binding at higher concentrations; the IC_{50} values were both approximately 80 μM. Guanosine phosphates, such as GTP, GDP and GMP, appeared to also inhibit the [^{32}P]cADPR binding to lesser extents at more than 1 mM. However, the other nucleotides tested did not have an inhibitory effect at the concentration of 1 mM.

Since the anti-cADPR antiserum cross-reacted to NAD^+, NMN^+, and guanosine phosphates, which are possibly present in many types of cells and tissues at millimolar or submillimolar concentrations, elimination of these nucleotides was required for precise determination of the cellular cADPR content with the RIA. It was noted that adenosine or guanosine had no inhibitory effect on the [^{32}P]cADPR binding to the antiserum (see the legend to Fig. 1). Thus, enzymic conversion of the

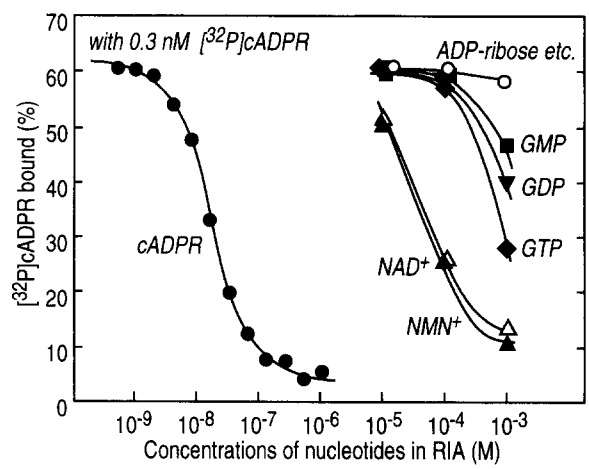

Fig. 1. Specificity of the anti-cADPR antiserum for various nucleotides. Anti-cADPR antiserum was incubated with 0.3 nM [^{32}P]cADPR in a reaction mixture (30 μl) containing various nucleotides at the final concentrations indicated on the *abscissa*, and [^{32}P]cADPR bound to the antiserum was then measured as described in section 2.3. The [^{32}P]cADPR binding to the antiserum was not inhibited by any other compound at concentrations of less than 1 mM. The tested compounds comprised ADP-ribose, ATP, ADP, AMP, adenosine, adenine, guanosine, IMP, UTP, UDP, CTP, TDP, cAMP, dibutyryl cAMP, cGMP, thymidine, and phosphoribose.

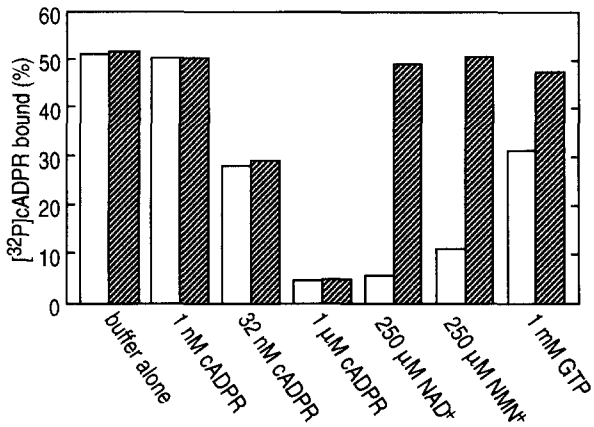

Fig. 2. Conversion of nucleotides that cross-reacted with the antiserum into inactive compounds. Nucleotides that cross-reacted with the anti-cADPR antiserum were first incubated at 25°C for 2 h with (hatched columns) or without (open columns) 50 U/ml of alkaline phosphatase and 2 U/ml of phosphodiesterase in 100 mM imidazole-HCl (pH 7.5), 100 mM NaCl, 400 mM KCl, and 2 mM $MgCl_2$ and then subjected to the standard RIA for cADPR as described in section 2.3. The final concentrations of nucleotides are indicated in the panel.

cross-reactive nucleotides into inactive compounds was performed with alkaline phosphatase and phosphodiesterase. This enzyme treatment results in the conversion of NAD^+ into nicotinamide ribonucleoside and adenosine via NMN^+ and adenosine phosphates as well as guanosine phosphates into guanosine. As shown in Fig. 2, this treatment was indeed effective for abolishing the inhibitory actions of NAD^+, NMN^+, and GTP. GDP and GMP were also inactivated by the enzyme treatment (data not shown). On the other hand, cADPR was resistant to these enzymes. Thus, more than 5 nM cADPR (i.e. 150 fmol in a 30-μl assay tube) could be detected reproducibly without any interference by the other nucleotides so far tested.

3.2. *Accumulation of immunoreactive cADPR in HL-60 cells caused to differentiate by RA*

We measured cADPR content in RA-treated HL-60 cells by means of the RIA, since the differentiated cells expressed CD38, which had enzyme activities of NADase and ADP-ribosyl cyclase [27]. As shown in Fig. 3A, CD38 mRNA was specifically induced by RA in accordance with our previous report [22]; HL-60 cells that had been caused to differentiate by other inducers or undifferentiated cells did not express CD38 mRNA. When cADPR was extracted with TCA from these cells and measured by the RIA (Fig. 3B), only the RA-treated cells were found to contain a high concentration of the cyclic nucleotide (138 ± 30 pmol/10^8 cells).

To confirm the increase in the cADPR content measurable by the RIA in RA-treated HL-60 cells, a TCA extract from the cells was analyzed by AG MP-1 column chromatography. The fractions eluted from the column were treated with alkaline phosphatase and phosphodiesterase and then subjected to RIA for cADPR. As shown in Fig. 4, immunoreactive cADPR was eluted as a single peak at the same retention time as the standard [^{32}P]cADPR. Moreover, the fraction containing immunoreactive cADPR exhibited Ca^{2+}-releasing activity, when it was analyzed with the microsomal fraction of sea urchin eggs (data not shown). More than 95% of the total immunoreactive cADPR applied to the column was recovered at the correct elution position of cADPR, indicating that the present RIA for cADPR was reliable in terms of its specificity.

3.3. *A possible involvement of CD38 in the formation of cellular cADPR in leukemic cells*

The time courses of induction of CD38-NADase and cellular cADPR accumulation were next investigated after the addition of RA to HL-60 cells. As shown in Fig. 5, there was a rapid increase in CD38-NADase activity of plasma membrane in accordance with our previous report [22]; a significant level of the enzyme activity was observed at 12 h, and the activity reached a maximum level at 24 h. On the other hand, cellular cADPR increased gradually during the first 24-h culture and then rapidly after 30 h, and a high cADPR content was maintained during the following culture with RA. These results, together with the data in Fig. 3, suggested that increase in cellular cADPR content is resultant from the expression of CD38 mRNA (and the induction of the ecto-enzyme of CD38) under the present conditions. Thus, we further investigated relationship between the expression level of CD38 mRNA and cellular cADPR accumulation in other types of cells.

As shown in Fig. 6A, the expression of CD38 mRNA was constitutively observed in MOLT-4 cells, a human acute lymphoblastic leukemia cell line. This cell line contained a high level of cellular cADPR (Fig. 6B. On the other hand, THP-1 cells, a monocytic leukemia cell line, contained a low level of CD38 mRNA. However, there was an increase in CD38 mRNA level after the cells were incubated with RA for 2 days. The cellular cADPR concentration in the RA-treated THP-1 cells was higher than that in the non-treated ones. Thus, the increase in CD38 mRNA appeared to lead the cellular cADPR accumulation of cells.

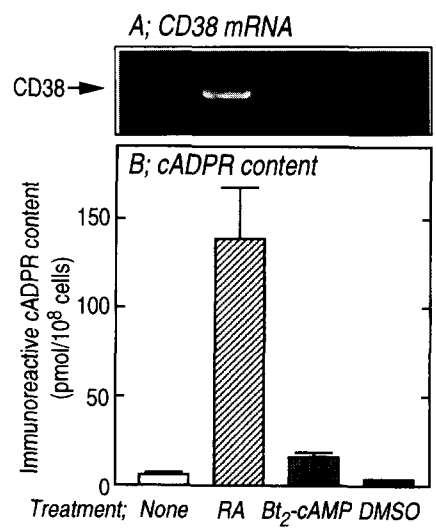

Fig. 3. Expression of CD38 mRNA and the cellular cADPR content in HL-60 cells cultured with various reagents. HL-60 cells were cultured without (*None*) or with 1 μM RA (*RA*), 0.5 mM dibutyryl cAMP (*Bt_2-cAMP*), or 1.2% dimethyl sulfoxide (*DMSO*) for 40 h. The cells were washed with Krebs-Ringer buffer (*minus* Ca^{2+}) and then subjected for the assaying of CD38 mRNA (panel A) and the cADPR content (panel B). CD38 mRNA was analyzed by PCR as described previously [22]. The cADPR content was measured by RIA, and mean values obtained for three separate experiments are shown with S.E.M.

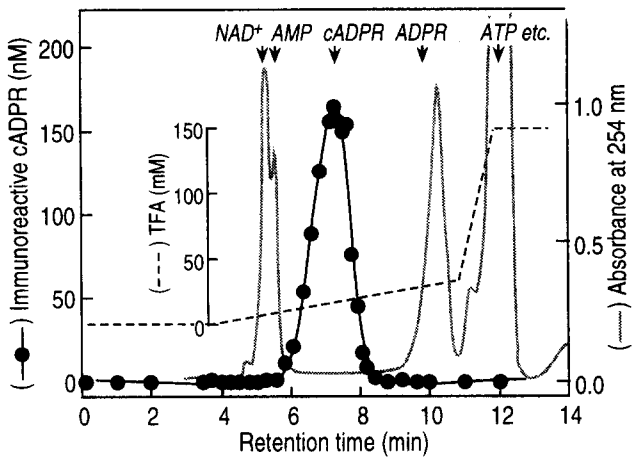

Fig. 4. Separation of immunoreactive cADPR by AG MP-1 column chromatography. Nucleotides were extracted with TCA from HL-60 cells (2.5×10^8 cells) which had been cultured with 1 μM RA for 40 h and separated by means of AG MP-1 column chromatography as described in section 2.4. Fractions eluted from the column, after being treated with alkaline phosphatase and phosphodiesterase, were subjected to the standard RIA for cADPR (●). The absorbance at 254 nm of eluted nucleotides was also monitored (------). The elution positions of standard nucleotides separated under the same conditions are indicated by arrows.

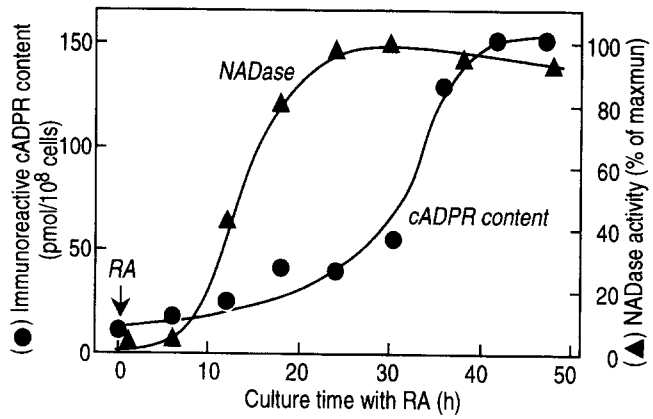

Fig. 5. Time courses of the induction of NADase and the increase in the cellular cADPR content in RA-treated HL-60 cells. HL-60 cells were cultured with 1 μM RA for the indicated times, and then the cADPR content (●) in the cells was measured by RIA. Membrane fractions were also isolated from the cultured cells for the assaying of NADase activity (▲) as described in section 2.5. The activity is expressed as a percentage of the maximum value, which was 60 nmol of nicotinamide formation/min/mg of protein.

4. Discussion

In the present study, we succeeded in developing a RIA method for measurement of the cellular cADPR content, by which more than a hundred fmol of the cyclic nucleotide could be specifically detected. The antiserum used in the present RIA exhibited high specificity for cADPR and very weak cross-reactivity to other nucleotides, such as NAD^+ and NMN^+ (see Fig. 1). The interference by NAD^+ and NMN^+ was eliminated by enzyme treatment of the test samples with alkaline phosphatase and phosphodiesterase, which converted the cross-reactive nucleotides into inactive compounds (see Fig. 2). This treatment will be applicable not only to the cultured cells investigated in the present study but also to a wide variety of biological samples. Indeed, our preliminary results with the present RIA indicated that rat brain contains approximately 200 nM cADPR under resting conditions (data not shown), which is the same value as that obtained by Lee et al. by means of a Ca^{2+}-releasing assay [11]. Moreover, there was 20–200 nM immunoreactive cADPR in various tissues isolated from rats.

We first applied this RIA system to the measurement of the cellular cADPR content in RA-treated HL-60 cells, since RA specifically induced CD38-NADase [22], which also catalyzes cADPR formation from NAD^+, but with lower specific activity [27]. Our present findings clearly indicate that there was a more than 20-fold increase in the cellular cADPR content of the differentiated HL-60 cells (see Fig. 3). If the cADPR had been homogeneously distributed in the cytoplasmic space of cells with a cell volume of 2 pl, the cellular concentration of the nucleotide would be approximately 1 μM.

Our present results indicated that the expression of CD38 mRNA resulted in increase in the cellular cADPR content in several types of cells. However, it is not apparent how the ecto-form enzyme of CD38 interacts with NAD^+ to generate cADPR inside cells. Our preliminary data suggested that the accumulated cADPR was not present at least in the plasma membranes of HL-60 cells and that activity of CD38-NADase could not be detected in the cytoplasmic fraction of the cells (data not shown). At the present time, we can not totally rule out the possibility that an enzyme(s) other than CD38 is involved in the synthesis of cellular cADPR. Further studies would be required to elucidate the significance of CD38 as an effective enzyme to produce intracellular cADPR. In any case, the present paper is the first showing that there is a significant change in the cellular cADPR content upon the differentiation of mammalian cells. The RIA for cADPR developed in the present study will facilitate studies on the physiological role of the new cyclic nucleotide in signal transduction.

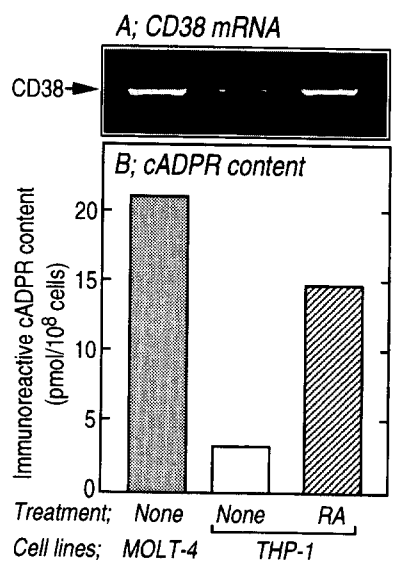

Fig. 6. High levels of cellular cADPR content in cultured cells expressing CD38 mRNA. MOLT-4 and THP-1 cells were cultured with (*RA*) or without (*None*) 1 μM RA for 48 h and then subjected for assaying of CD38 mRNA (panel A) and the cellular cADPR content (panel B) as described in Fig. 3.

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