All-trans-retinoic acid stimulates synthesis of cyclic ADP-ribose in renal LLC-PK1 cells.

K W Beers, … , E N Chini, T P Dousa

J Clin Invest. 1995;95(5):2385-2390. https://doi.org/10.1172/JCI117932.

Research Article

Cyclic adenosine diphospho-ribose (cADPR) triggers Ca2+ release from intracellular stores and is therefore proposed to function as a second messenger in cellular signaling; however, an extracellular stimulus, i.e., first messenger (hormone or autacoid) that modulates cADPR metabolism has not been identified. We discovered that all-trans-retinoic acid (atRA) is a potent stimulus to increase cADPR synthesis by cultured LLC-PK1 cells. The stimulation of cADPR synthesis by atRA is dose dependent between 0.1 nM and 1 microM (maximum increase approximately delta + 600%), while atRA does not alter the rate of cADPR hydrolysis by LLC-PK1 cells. The activity of other intrinsic apical membrane enzymes was not significantly altered. The stimulation of cADPR synthesis by atRA occurs after a lag period of 6-8 h, and the stimulation is inhibited by actinomycin D and by cycloheximide. Our results therefore demonstrate that atRA in physiological concentrations is a potent extracellular stimulus, first messenger, that enhances cADPR synthesis, and the effect of atRA requires de novo protein synthesis. We suggest that some of the diverse biologic actions of atRA such as morphogenetic and cell differentiation may be mediated via cADPR.

All-*trans*-Retinoic Acid Stimulates Synthesis of Cyclic ADP-Ribose in Renal LLC-PK$_1$ Cells

Kelly W. Beers, Eduardo N. Chini, and Thomas P. Dousa
Nephrology Research Unit, Division of Nephrology and Internal Medicine, Departments of Medicine and Physiology/Biophysics, Mayo Clinic and Foundation, Mayo Medical School, Rochester, Minnesota 55905

Abstract

Cyclic adenosine diphospho-ribose (cADPR) triggers Ca^{2+} release from intracellular stores and is therefore proposed to function as a second messenger in cellular signaling; however, an extracellular stimulus, i.e., first messenger (hormone or autacoid) that modulates cADPR metabolism has not been identified. We discovered that all-*trans*-retinoic acid (atRA) is a potent stimulus to increase cADPR synthesis by cultured LLC-PK$_1$ cells. The stimulation of cADPR synthesis by atRA is dose dependent between 0.1 nM and 1 μM (maximum increase $\sim \Delta + 600\%$), while atRA does not alter the rate of cADPR hydrolysis by LLC-PK$_1$ cells. The activity of other intrinsic apical membrane enzymes was not significantly altered. The stimulation of cADPR synthesis by atRA occurs after a lag period of 6–8 h, and the stimulation is inhibited by actinomycin D and by cycloheximide. Our results therefore demonstrate that atRA in physiological concentrations is a potent extracellular stimulus, first messenger, that enhances cADPR synthesis, and the effect of atRA requires de novo protein synthesis. We suggest that some of the diverse biologic actions of atRA such as morphogenetic and cell differentiation may be mediated via cADPR. (*J. Clin. Invest.* 1995. 95:2385–2390.) Key words: cyclic adenosine diphospho-ribose • cADPR • retinoic acid • LLC-PK$_1$ cells • signaling

Introduction

Cyclic adenosine diphospho-ribose (cADPR),[1] a metabolite of β-NAD$^+$, is a potent agent that triggers release of Ca^{2+} from intracellular stores in the sea urchin egg (1) and eukaryotic cells (2) by a mechanism that is analogous to the Ca^{2+}-releasing action of inositol 1,3,4-triphosphate (IP_3). However, the action of cADPR differs from IP_3 in that cADPR modulates Ca^{2+} efflux through the ryanodine channel and regulates Ca^{2+}-induced Ca^{2+} release (3, 4). The membrane-bound enzymes that synthesize cADPR from β-NAD$^+$, (ADP-ribosyl cyclase [ADPR cyclase]) and hydrolyze cADPR to ADP-ribose (cADPR-hydrolase) are found in many tissues and cell types (5). However, unlike IP_3, which via intracellular Ca^{2+} release serves as a second messenger of many hormones or autacoids (6), no extracellular hormonal first messenger has yet to be identified that modulates cADPR synthesis and(or) accumulation.

An early observation by Hemmi and Breitman (7) that was recently reproduced (8) demonstrated that incubation of the human promyelocytic cell line, HL-60, with all-*trans*-retinoic acid (atRA), a hormone known to possess both differentiation and morphogenetic activity, is accompanied by a dramatic increase in NAD$^+$-glycohydrolase. Furthermore, NAD$^+$-glycohydrolases isolated from diverse sources: dog spleen, *Bungarus fascictus* snake venom (9), or human erythrocytes (10), can catalyze not only NAD$^+$ hydrolysis to ADP-ribose (ADPR), but also synthesis of cADPR from β-NAD$^+$ and hydrolysis of cADPR to ADPR (9, 10). Based on these reports, we propose that atRA may also serve as a hormonal stimulus, first messenger, for ADPR cyclase to augment the generation of cADPR from β-NAD$^+$ (Fig. 1). This hypothesis was tested on renal LLC-PK$_1$ epithelial cells, a well established model for study of cell signaling and regulation of epithelial transport (11), which also contain a cellular retinoid-binding protein (12). We have found that atRA is a specific and potent stimulus of cADPR synthesis in LLC-PK$_1$ cells.

Methods

According to previous reports, NAD$^+$-glycohydrolase, which also catalyzes cADPR metabolism (9, 10), is predominantly an ectoenzyme in mammalian tissues (13), including the kidney (14). Therefore, the rate of cADPR formation and hydrolysis by LLC-PK$_1$ cells was determined by incubating intact cell monolayers with requisite substrates.

Cells. LLC-PK$_1$ cells (passages 45–60) from American Type Culture Collection (Rockville, MD) were grown in Dulbecco's modified Eagle's medium (DME) containing Ham's F-12 nutrient mixture and 0–10% fetal calf serum (FCS) with the addition of an antimycotic solution consisting of 100 U/liter penicillin, 100 μg/liter streptomycin, and 0.25 μg/liter amphotericin B (GIBCO BRL, Gaithersburg, MD). Cell cultures were grown and maintained in 75-cm^2 plastic flasks in a humidified incubator supplied with 5% CO_2/95% air at 37°C. Subcultures were obtained as needed by detaching the cells with a Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) solution containing 0.25% trypsin

This work was presented in part at the 27th annual meeting of the American Society of Nephrology in Orlando, FL, on 28 October 1994.

Address correspondence to Thomas P. Dousa, M.D., 921B Guggenheim Building, Mayo Clinic, Rochester, MN 55905. Phone: 507-284-4343; FAX: 507-284-8566.

Received for publication 21 October 1994 and in revised form 9 January 1995.

1. *Abbreviations used in this paper:* Act D, actinomycin D; ADPR cyclase, ADP-ribosyl cyclase; atRA, all-*trans*-retinoic acid; cADPR, cyclic adenosine diphospho-ribose; cADPR hydrolase, cADPR glycohydrolase; 13-*cis*-RA, 13-*cis*-retinoic acid; ECM, extracellular medium; IP_3, inositol 1,3,4-triphosphate; RAR, retinoic acid receptor.

The Journal of Clinical Investigation, Inc.
Volume 95, May 1995, 2385–2390

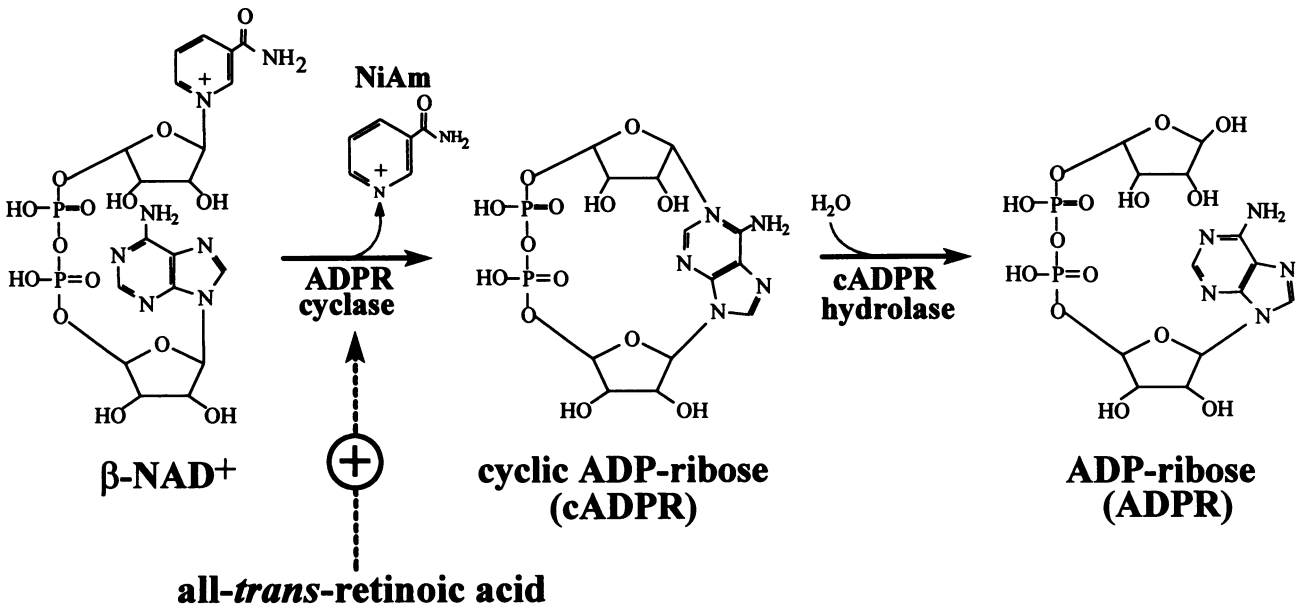

Figure 1. Pathway of cADPR metabolism and proposed site of action of atRA.

and 5 mM EDTA. For experiments, the cells were seeded into 6-well plastic plates (9.6 cm^2/well) at a density of 100,000 cells/well.

Quiescent cells. In preliminary experiments we observed that the atRA-elicited stimulation of cADPR synthesis was greatly amplified in subconfluent, quiescent cells. Therefore, unless otherwise stated the following was standard procedure. LLC-PK_1 cells were grown in DME medium containing 10% FCS until they reached ~ 70% confluency and then were made quiescent by replacing the growth medium with medium containing only 0.5% FCS for 48 h. Cell medium was replaced again with FCS-free medium that contained atRA (or other tested agents solubilized in 1% DMSO) added to the final concentrations as specified in Results for indicated time periods. Control treated cells were exposed only to medium containing 1% DMSO.

ADPR cyclase activity. Quiescent LLC-PK_1 cells preincubated with atRA or other retinoids for various time periods, as specified in the Results, were thoroughly rinsed with PBS. Thereafter, the cells were incubated at 37°C in an extracellular medium (ECM) of the following composition (final concentration): 0.5 mM β-NAD^+, 65 mM NaCl, 20 mM Hepes, 20 mM D-glucose adjusted to pH 7.4 with Tris base. At the end of the incubation period, as specified in Results, aliquots of the incubation medium were removed and cADPR content in the sample was determined by a specific Ca^{2+}-release bioassay.

cADPR hydrolase activity. The hydrolysis of cADPR was determined analogously to ADPR cyclase, except that after preincubation with atRA, the cells were incubated in ECM containing 10 μM cADPR as substrate, instead of β-NAD^+. During incubation with cADPR, aliquots of medium were removed at 1, 2, and 4 h and assayed for cADPR concentration remaining in the cell medium using the Ca^{2+}-release bioassay as described (5). cADPR hydrolase activity of atRA incubated cells was compared with that of control treated cells.

Purification of β-NAD^+. Before assaying cells for ADPR cyclase activity, trace amounts of naturally occurring cADPR were removed from commercial β-NAD^+ substrate by anion-exchange HPLC as previously described (1). Purified β-NAD^+ was then Speed-vac concentrated to 15 mM and pH adjusted to 7.4 with Ca^{2+}-free Tris base.

Sea urchin egg homogenate. Synthesis and hydrolysis of cADPR by LLC-PK_1 cells were measured by a specific Ca^{2+}-release bioassay that was prepared as described previously (3, 5, 15). Briefly, eggs collected from *Lytechinus pictus* sea urchins (Marinus Inc., Long Beach, CA) were dejellied by sequential washings (1,000 *g*, 60 s) in Ca^{2+}-free artificial sea water containing 1 mM EGTA, then in Ca^{2+}-free artificial sea water without EGTA, followed by two washes in an intracellular medium containing 250 mM *N*-methylglucamine, 250 mM K^+ gluconate, 20 mM Hepes, and 1 mM $MgCl_2$ (pH 7.2 adjusted with acetic acid) and resuspended to 25% (vol/vol) with intracellular medium. Protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 50 μg/ml soybean trypsin inhibitor) and an ATP-regenerating system composed of 2 U/ml creatine kinase, 4 mM phosphocreatine, and 0.5 mM ATP were added and the eggs were homogenized with a Dounce-type homogenizer. The homogenate was centrifuged (13,000 *g*, 4°C) for 10 s and the collected supernatant was aliquoted and stored at −70°C.

cADPR bioassay. Aliquots of LLC-PK_1 cell medium (3–6 μl) were added to a cuvette containing 250 μl of sea urchin egg homogenate that was maintained at 17°C with constant stirring in a fluorescence spectrophotometer (model F2000; Hitachi Instruments, Inc., San Jose, CA). Ca^{2+} release from sea urchin egg homogenates was monitored by Fluo-3 fluorescence changes. The quantum of cADPR synthesized by LLC-PK_1 cells was determined by comparing Fluo-3 fluorescence in aliquots of cell medium to standards of authentic cADPR. The concentration of cADPR in the cuvette was then converted to nanomoles per milligram of cell protein based on the amount of protein per well and the final dilution factor. The protein content in each well was determined by the method of Lowry et al. (16) after solubilization of cells in 0.5% dodecylsulphate.

HPLC identification of cADPR. Media from control and atRA-treated LLC-PK_1 cells incubated with β-NAD^+ were analyzed for cADPR content by anion-exchange chromatography as described previously (1) with minor modifications. AG MP-1 resin (Bio-Rad, Hercules, CA) was hand packed into a 6.6 × 250–mm column and the nucleotides were eluted with the following nonlinear gradient of 150 mM trifluoroacetic acid in water. At a constant flow rate of 4 ml/min, solvent B (150 mM trifluoroacetic acid) was initiated at 0.7% and held for 1 min. Solvent B was then increased to 2% from 1 to 3 min and held until 6 min, then linearly increased to 10% from 6 to 15 min. At 15 min, solvent B was increased to 100% over 0.1 min and held for 5 min, then decreased within 0.1 min to the starting conditions of 0.7%. cADPR detected by UV_{260} absorption eluted at 13.5 min under the conditions of 8.7% solvent B (see Fig. 4 *A*).

DNA synthesis in LLC-PK_1 cells. Incorporation of [^{3}H]thymidine into DNA of LLC-PK_1 cells was performed using standard procedures

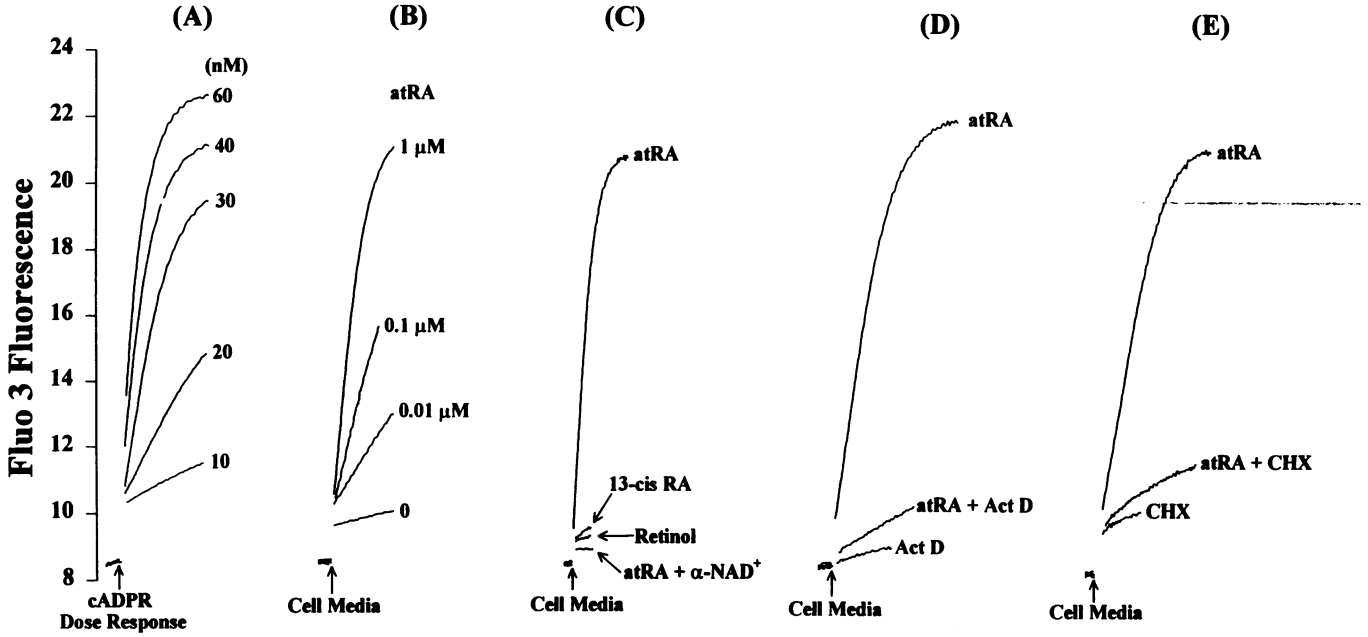

Figure 2. Stimulation of cADPR synthesis in LLC-PK$_1$ cells treated with atRA. (*A*) Calibration curve with authentic cADPR added to the sea urchin egg bioassay (1). (*B*) Effect of 24 h of incubation with various concentrations of atRA upon the synthesis and accumulation of cADPR by LLC-PK$_1$ cells. (*C*) Effect of incubation with other retinoids upon cADPR synthesis in LLC-PK$_1$ cells. All retinoids were tested at a concentration of 1 μM; 13-*cis*-RA = 13-*cis*-retinoic acid. Also shown is the effect of replacing 0.5 mM β-NAD$^+$ substrate with 0.5 mM α-NAD$^+$ upon cADPR synthesis by atRA-treated LLC-PK$_1$ cells. (*D*) Effect of 1 μg/ml Act D on synthesis of cADPR. LLC-PK$_1$ cells were first preincubated with or without Act D for 3 h in FCS-free medium. Medium was then changed to remove Act D, and the cells were then incubated for 12 h in the FCS-free medium containing 1 μM RA, then assayed for ADPR cyclase activity. (*E*) Effect of 40 μg/ml cycloheximide (*CHX*) on cADPR synthesis. Experimental procedures to test the effect of CHX were exactly as described for the Act D–treated cells. Tracings are from a representative experiment, repeated three times. Abscissa: fluorescence of Fluo-3 in arbitrary units.

(17, 18). Briefly, cells were pulsed with 1 μCi/well [^{3}H]thymidine (24 Ci/mmol) during the last 4 h of incubation with atRA, then washed with PBS, lysed with 0.2 N NaOH, and neutralized with 1 N HCl and 10% TCA. The material was then transferred to a GF/C filter (Whatman Inc., Clifton, NJ) previously placed in a cell harvester. The filter was washed once with TCA, then twice with 100% ethanol and [^{3}H]thymidine determined by standard liquid scintillation counting.

Apical membrane–bound enzyme assays. The activities of alkaline phosphatase, γ-glutamyltransferase, and leucine aminopeptidase were measured as follows. Quiescent LLC-PK$_1$ cells were scraped from plastic plates, washed twice, and resuspended in a NaCl buffer containing 137 mM NaCl and 20 mM Hepes/Tris at 37°C, pH 7.4. The cells were then subjected to two freeze/thaw cycles, pelleted and resuspended in hypotonic medium (10 × dilution of the NaCl medium), and then passed through a 26-gauge needle six times. The fractionated membranes were then pelleted and resuspended in the NaCl buffer and assayed for enzyme activity as we have described earlier (19).

Statistical analysis. When appropriate, the results were analyzed statistically using group *t* tests.

Reagents. Fluo-3 was obtained from Molecular Probes (Eugene, OR). [^{3}H]Thymidine was acquired from DuPont/New England Nuclear (Boston, MA). IP_3, β-NAD$^+$, α-NAD$^+$, ADP-ribose, and all other chemicals of the highest purity available were purchased from Sigma Chemical Co. (St. Louis, MO). Authentic cADPR was in part a gift from Dr. Hon Cheung Lee (Department of Physiology, University of Minnesota, Minneapolis, MN) or purchased from Amersham Corp. (Arlington Heights, IL).

Results and Discussion

When quiescent, subconfluent LLC-PK$_1$ cell monolayers were preincubated with 10 nM atRA for 24 h, then tested for ADPR cyclase activity, the cADPR accumulation in the ECM was increased nearly twofold over the control level (Fig. 2 *B* and Fig. 3 *A*). The stimulatory effect of atRA was also time dependent (Fig. 3 *B*) and increased with atRA concentration reaching a maximum (Δ + 600±50%; $P < 0.01$, $n = 12$) at 1 μM atRA (Fig. 2 *B* and Fig. 3 *A*). In sharp contrast, when LLC-PK$_1$ cells were grown to full confluency before serum deprivation, atRA even at the maximum dosage of 1 μM was distinctly less effective (maximum Δ + 200%) in stimulating cADPR accumulation (not shown) than in subconfluent cells (Fig. 3 *A*).

The elevated cADPR content in LLC-PK$_1$ cell medium elicited by atRA was not due to decreased cADPR hydrolysis. When control or atRA-pretreated LLC-PK$_1$ cells were incubated with 10 μM cADPR as substrate for 1, 2, or 4 h, the rate of decrease in cADPR concentration in the cell medium (data not shown) did not differ, indicating that atRA does not alter cADPR-hydrolase activity in LLC-PK$_1$ cells. Under these conditions, the hydrolase activity was quite low compared with that of human red blood cells (10); cADPR concentration had decreased by only ~ 10% for either treatment after 4 h of incubation. These findings suggest that the atRA-elicited accumulation of cADPR is due solely to an increase in cADPR synthesis, i.e., by enhancing activity of ADPR cyclase while not affecting cADPR hydrolase.

The metabolite of β-NAD$^+$ was verified to be cADPR by both HPLC analysis and homologous desensitization of the bioassay. A summary of HPLC chromatograms is shown in Fig. 4 that compares the elution profile of authentic cADPR and related nucleotides (Fig. 4 *A*) to media from control or atRA-

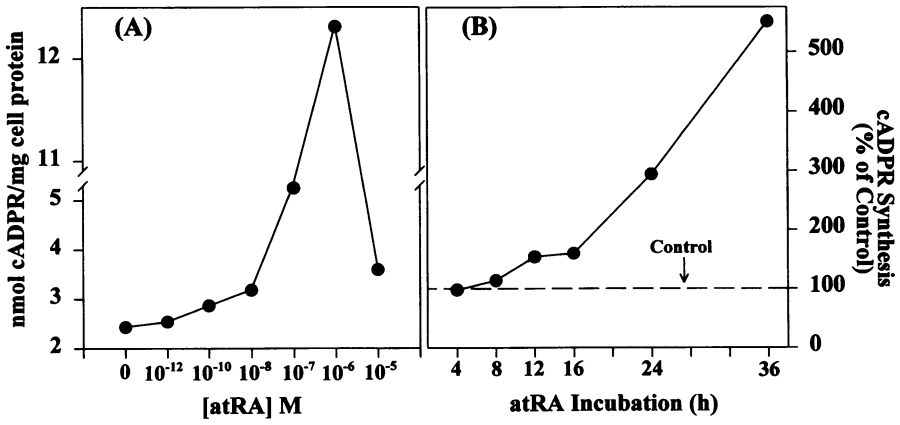

Figure 3. Dose dependency (*A*) and time course (*B*) of atRA stimulation of cADPR synthesis in LLC-PK$_1$ cells. (*A*) LLC-PK$_1$ cells were incubated with various concentrations of atRA for 24 h, then rinsed, and ADPR cyclase was assayed as described in Methods. (*B*) LLC-PK$_1$ cells were incubated with 1 μM atRA for various time periods; at the end of each period, cells were rinsed and ADPR cyclase activity was determined (see Methods). The stimulation of cADPR synthesis by atRA is expressed as percent difference from control (no atRA added) for each time period. Data presented are a representative experiment, repeated three times.

pretreated cells. As shown (Fig. 4, *B* and *C*), media from control and atRA cells contain a compound that elutes at precisely the same time as authentic cADPR. Moreover, Ca^{2+} release activity of the cell medium, as tested by the bioassay, was localized exclusively to the peak corresponding to cADPR. Based on the area under the HPLC peaks, cADPR concentration in the medium of atRA-pretreated cells was over twofold greater than in control cell medium.

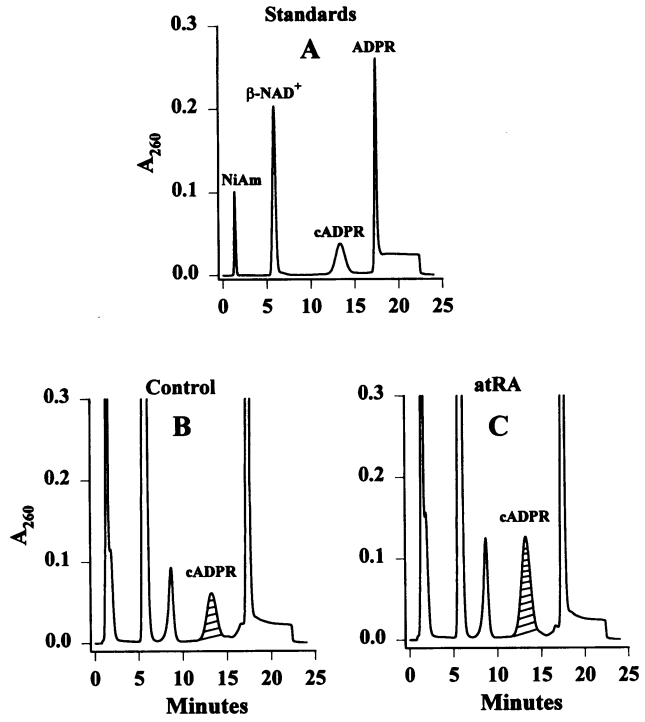

Figure 4. HPLC separation and identification of cADPR synthesized by LLC-PK$_1$ cells. Cells were exposed to vehicle (*Control*) or atRA (1 μM) for 24 h and then incubated in a buffer (20 mM Tris-HCl and 20 mM glucose; pH 7.4) containing 1 mM β-NAD$^+$. After 8 h of incubation, the medium was removed, centrifuged (10,000 *g*, 10 min) to remove any cell debris, and separated by anion-exchange HPLC as described in Methods. *A* displays the elution profile of authentic standards. For comparison, the concentration of each indicated standard (NiAm is nicotinamide) is 20 nmol. *B* and *C* exhibit the synthesis of cADPR by cells under control conditions or atRA pretreated cells, respectively. Ca^{2+}-release activity of the eluate, as tested by the bioassay, corresponds exclusively to the shaded peak depicted in *B* and *C*.

Further verification that cADPR was synthesized by LLC-PK$_1$ cells was provided by evidence of homologous desensitization of the bioassay (Fig. 5). The use of this technique to determine identity of Ca^{2+}-releasing compounds in sea urchin egg homogenate is well established (1, 20). In the sea urchin egg bioassay, repeat additions of the same Ca^{2+}-releasing compound, e.g., cADPR or IP_3, will homologously desensitize that particular Ca^{2+} release system, while the homogenate remains sensitive to other compounds. Indeed, the bioassay is homologously desensitized to atRA-treated cell medium, but not to IP_3, after repeat additions of authentic cADPR (Fig. 5 *A*). The reverse sequence of additions will also produce the same response, in that atRA-treated cell medium will desensitize the bioassay to authentic cADPR, but not to IP_3 (Fig. 5 *B*). Conversely, the bioassay system desensitized to IP_3 still responds by Ca^{2+} release to atRA-treated LLC-PK$_1$ cell medium (Fig. 5 *C*). Replacement of β-NAD$^+$ by an equimolar concentration of α-NAD$^+$ as substrate for ADPR cyclase exemplifies the specific requirement for the β-glycosidic bond of NAD$^+$ by ADPR cyclase (1), since no accumulation of cADPR was observed (Fig. 2 *C*), another argument for authenticity of cADPR.

Preincubation of LLC-PK$_1$ cells with actinomycin D (Act D) or cycloheximide, to inhibit DNA or protein synthesis, respectively, completely blocked the atRA-elicited increase in cADPR accumulation (Fig. 2, *D* and *E*). This indicates that proteosynthesis is required for atRA-stimulated ADPR cyclase and that atRA presumably acts by binding to a cognate nuclear retinoic acid receptor (RAR) inducing activation (or inhibition) of specific genes (21). In another experiment, LLC-PK$_1$ cells were preincubated without or with 1 μM atRA for only 6 h, a period of time not sufficient enough to enhance cADPR synthesis (Fig. 3 *B*), then washed free of extracellular atRA and incubated in 0.5% FCS medium for an additional 72 h before assaying for ADPR cyclase activity. Under these test conditions, 72 h after atRA exposure, LLC-PK$_1$ cells synthesized nearly fivefold more cADPR than parallel tested control cells. These data are also consistent with the notion that atRA increases cADPR generation by a genomic mechanism, such as stimulating de novo synthesis of ADPR cyclase. However, alternative interpretations should be considered. atRA may stimulate synthesis of some unknown activator or may suppress synthesis of an inhibitor of ADPR cyclase. The exact mechanism of atRA in this action needs to be delineated in future studies; neverthe-

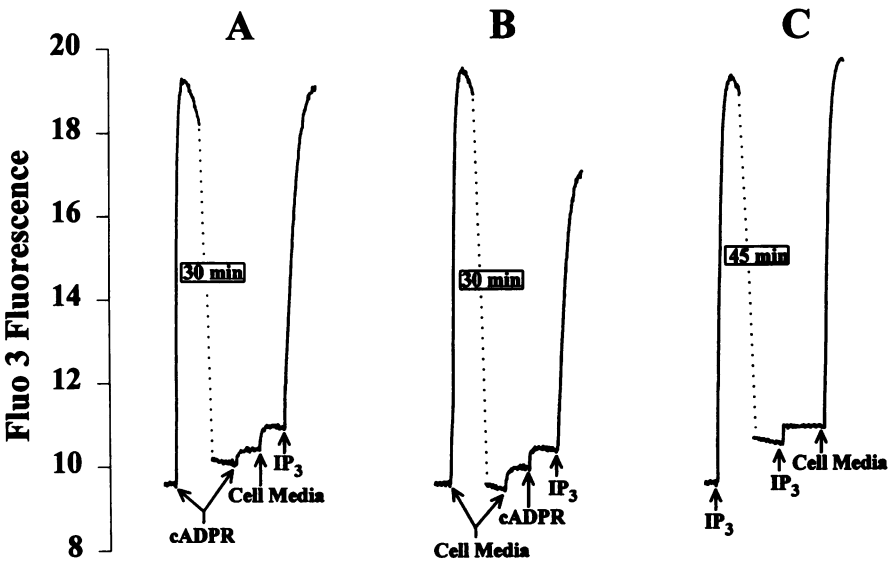

Figure 5. Homologous desensitization of the Ca^{2+}-release system in sea urchin egg homogenate. (*A*) After response to 80 nM cADPR and reuptake of Ca^{2+}, the subsequent additions of authentic cADPR or medium from LLC-PK_1 cells (further *Cell Media*) preincubated with atRA failed to produce a response, but 1 μM IP_3 triggered Ca^{2+} release. (*B*) After Ca^{2+}-release response to cell medium and Ca^{2+} reuptake, the bioassay was refractory to both cell medium and authentic cADPR, but responded to IP_3. (*C*) After Ca^{2+} release by IP_3 and reuptake, the bioassay was resistant to IP_3 but not to cell medium. Abscissa: fluorescence of Fluo-3 in arbitrary units.

less, our current results clearly demonstrate that the stimulatory effect of atRA upon cADPR synthesis depends on intact DNA and protein synthetic systems.

As in some other cell systems (18), atRA stimulates mitogenic DNA synthesis in LLC-PK_1 cells (17) and it should be considered if the amplified capacity for cADPR synthesis may be merely a reflection of the enhanced mitotic activity. Two findings argue against this possibility. First, incubation of quiescent LLC-PK_1 cells with 10% FCS stimulates [^{3}H]thymidine incorporation to a similar degree as 1 μM atRA (594±132 vs 393±72%) compared with control cells. Yet, unlike cells incubated with atRA in FCS-free medium, LLC-PK_1 cells incubated with 10% FCS show no increase in cADPR accumulation (Fig. 6). Secondly, in LLC-PK_1 cells treated with atRA, activities of enzymes intrinsic to the apical membrane such as alkaline phosphatase, γ-glutamyltransferase, and leucine aminopeptidase were not increased while ADPR cyclase in the same cells was markedly (Δ + ~ 300%) stimulated (Table I).

The stimulatory effect upon cADPR synthesis appears to be specific for atRA compared with 13-*cis*-retinoic acid (13-*cis*-RA), a stereoisomer of atRA, which in equimolar concentrations did not stimulate ADPR cyclase in LLC-PK_1 cells (Fig. 2 *C*). This is also consistent with the view that atRA acts via the nuclear RAR receptor, since although 13-*cis*-RA has multiple biologic effects, it does not bind onto RAR (22). Likewise, 1 μM retinol did not stimulate ADPR cyclase (Fig. 2 *C*); this pre-precursor of atRA is metabolized by LLC-PK_1 primarily to retinoids other than atRA (12).

It should be pointed out that atRA stimulates cADPR synthesis in low nanomolar concentrations, which commensurate with reported (12, 23) atRA plasmal levels both in humans (15 nM) and rats (6 nM). This finding is also consistent with the hypothesis that atRA acts as a hormonal first-messenger to stimulate cADPR generation in epithelial cells.

NAD^+-glycohydrolase is predominantly an ectoenzyme in most systems; however, in some cases (13), including tubular epithelia (14), there is also evidence of intracellular NAD^+-glycohydrolase activity. Based on this analogy, it is reasonable to expect that at least a minor portion of cADPR is synthesized intracellulary. In this study we did not explore intracellular synthesis of cADPR; however, intracellular cADPR may play a mediatory role via Ca^{2+} release akin to the effects of IP_3. A second messenger role for cADPR such as this has been sug-

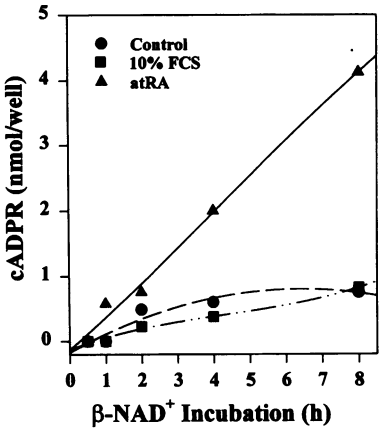

Figure 6. Specific stimulation of cADPR synthesis by atRA. Subconfluent LLC-PK_1 cells were incubated in a medium without FCS (*circles*) (control), with 1 μM atRA (*triangles*), or with 10% FCS and no atRA (*squares*) for 24 h. Then cells were thoroughly rinsed and incubated in an assay medium for ADPR cyclase assay containing 0.5 mM β-NAD^+. At indicated times, 4 μl of the cell medium was removed and tested for cADPR content using the bioassay. Data presented are from a representative experiment that was repeated four times.

Table I. Apical Membrane Enzymes in LLC-PK_1 Cells Incubated for 24 h with 1 μM atRA Compared with Controls (Media without FCS) or Cells Incubated in Media with 10% FCS

Condition	Alkaline phosphatase	γ-Glutamyl transferase	Leucine aminopeptidase	cADPR synthesis
		μmol/mg protein/min		*nmol/mg*
Control	303±40*	1461±141	85±11	3.7±0.4
atRA	358±64	1680±140	126±18	12.3±0.9‡
10% FCS	534±209	1566±358	131±38	2.5±0.4

* Each number denotes mean±SEM from five experiments. ‡ Denotes value significantly higher ($P < 0.01$; t test) than value for control or 10% FCS.

gested for insulin secretion from β-pancreatic cells (24) in response to increased D-glucose concentration.

Our finding that atRA stimulates cADPR synthesis at the cell surface and releases it into the ECM has highly attractive functional implications. It is conceivable that cADPR may serve as an intercellular paracrine nucleotide, second messenger of atRA that is operant in cell–cell communications. A function such as intercellular signaling would be, in principle, analogous to the role which cAMP plays in intercellular communication between eukaryotic cells leading to formation of cellular contacts as it was documented in detail for *Dictyosterlium discoideum* (25). atRA is an archetypal hormone that regulates morphogenesis, organogenesis, and differentiation (26). For example, atRA was shown to trigger tubulogenesis in rabbit renal epithelia (27) and induced formation of organoids in cultured glomerular SGE_1 epithelial cells when applied as retinol acetate (28). It is thus reasonable to surmise that atRA-stimulated synthesis of cADPR in renal epithelia and other tissue types may generate intercellular signals that coordinate functions and contacts of adjacent or distant cells in the course of morphogenesis and organogenesis.

In summary, we report herein that atRA is a potent stimulus of cADPR synthesis which most likely acts via a genomic mechanism. We thus propose that cADPR has the potential to mediate some of the multiple biologic effects of atRA.

Acknowledgments

We thank Mr. M. A. Thompson for expert technical assistance and Ms. Mary E. Bennett for excellent secretarial assistance.

This work was supported by National Institute of Diabetes, Digestive and Kidney Diseases grant DK-30759 and the Mayo Foundation. Dr. K. W. Beers is a recipient of a research fellowship from the National Institutes of Health (training grant DK-07013).

References

1. Rusinko, N., and H. C. Lee. 1989. Widespread occurrence in animal tissues of an enzyme catalyzing the conversion of NAD^+ into a cyclic metabolite with intracellular Ca^{2+}-mobilizing activity. *J. Biol. Chem.* 264:11725–11731.

2. Koshiyama, H., H. C. Lee, and A. H. Tashjian. 1991. Novel mechanism of intracellular calcium release in pituitary cells. *J. Biol. Chem.* 266:16985–16988.

3. Lee, H. C. 1993. Potentiation of calcium- and caffeine-induced calcium release by cyclic ADP-ribose. *J. Biol. Chem.* 268:293–299.

4. Galione, A., H. C. Lee, and W. B. Busa. 1991. Ca^{2+}-induced Ca^{2+} release in sea urchin egg homogenates: modulation by cyclic ADP-ribose. *Science (Wash. DC).* 253:1143–1146.

5. Lee, H. C., and R. Aarhus. 1993. Wide distribution of an enzyme that catalyzes the hydrolysis of cyclic ADP-ribose. *Biochim. Biophys. Acta.* 1164:68–74.

6. Cockcroft, S., and G. M. H. Thomas. 1992. Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. *Biochem. J.* 288:121–134.

7. Hemmi, H., and T. R. Breitman. 1982. Induction by retinoic acid of NAD^+-glycohydrolase activity of myelomonocytic cell lines HL-60-THP-1 and U-937, and fresh human acute promyelocytic leukemia cells in primary culture. *Biochem. Biophys. Res. Commun.* 109:669–674.

8. Kontani, K., H. Nishina, Y. Ohoka, K. Takahashi, and T. Katada. 1993. NAD glycohydrolase specifically induced by retinoic acid in human leukemic HL-60 cells. *J. Biol. Chem.* 268:16895–16898.

9. Kim, H., E. L. Jacobson, and M. K. Jacobson. 1993. Synthesis and degradation of cyclic ADP-ribose by NAD glycohydrolases. *Science (Wash. DC).* 261:1330–1333.

10. Lee, H. C., E. Zocchi, L. Guida, L. Franco, B. Umberto, and A. De Flora. 1993. Production and hydrolysis of cyclic ADP-ribose at the outer surface of human erythrocytes. *Biochem. Biophys. Res. Commun.* 191:639–645.

11. Ausiello, D. A., D. H. Hall, and J. M. Dayer. 1980. Modulation of cyclic AMP-dependent protein kinase by vasopressin and calcitonin in cultured porcine renal $LLC\text{-}PK_1$ cells. *Biochem. J.* 186:773–780.

12. Napoli, J. L. 1986. Retinol metabolism in $LLC\text{-}PK_1$ cells. *J. Biol. Chem.* 261:13592–13597.

13. Muller, H. M., C. D. Muller, and F. Schuber. 1983. NAD^+ glycohydrolase, an ecto-enzyme of calf spleen cells. *Biochem. J.* 212:459–464.

14. Kempson, S. A. 1985. NAD-glycohydrolase in renal brush border membranes. *Am. J. Physiol.* 249:F366–F373.

15. Clapper, D. L., T. F. Walseth, P. J. Dargie, and H. C. Lee. 1987. Pyridine nucleotide metabolites stimulate calcium release from sea urchin egg microsomes desensitized to inositol triphosphate. *J. Biol. Chem.* 262:9561–9568.

16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.

17. Mantzouris, N. M., J. Kaplan, G. Clark, and M. K. Hise. 1993. Regulation of epidermal growth factor receptor expression and growth by protein kinase C and retinoic acid in $LLC\text{-}PK_1$ cells. *Am. J. Kidney Dis.* 22:858–864.

18. Argiles, A., N. E. Kraft, P. Hutchinson, S. Senes-Ferrari, and R. C. Atkins. 1989. Retinoic acid affects the cell cycle and increases total protein content in epithelial cells. *Kidney Int.* 36:954–959.

19. Hoppe, A., J. T. Lin, M. Onsgard, F. G. Knox, and T. P. Dousa. 1991. Quantitation of the Na^+-P_i cotransporter in renal cortical brush border membranes. *J. Biol. Chem.* 266:11528–11536.

20. Howard, M., J. C. Grimaldi, J. F. Bazan, F. E. Lund, L. Santos-Argumedo, R. M. E. Parkhouse, T. F. Walseth, and H. C. Lee. 1993. Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science (Wash. DC).* 262:1056–1059.

21. Giguere, V. 1994. Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. *Endocrinol. Rev.* 15:61–79.

22. Armstrong, R. B., K. O. Ashenfelter, C. Eckhoff, A. A. Levin, and S. S. Shapiro. 1994. General and reproductive toxicology of retinoids. *In* The Retinoids—Biology, Chemistry and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, New York. 545–572.

23. Sporn, M. B., A. B. Roberts, and D. S. Goodman. 1994. The Retinoids—Biology, Chemistry and Medicine. Raven Press, New York. 679 pp.

24. Takasawa, S., K. Nata, H. Yonekura, and H. Okamoto. 1993. Cyclic ADP-ribose in insulin secretion from pancreatic β cells. *Science (Wash. DC).* 259:370–373.

25. Devreotes, P. N. 1983. Cyclic nucleotides and cell-cell communication in *Dictyostelium discoideum. In* Advances in Cyclic Nucleotide Research. P. Greengard, and G. A. Robison, editors. Raven Press, New York. 55–96.

26. Hofmann, C., and G. Eichele. 1994. Retinoids in development. *In* The Retinoids—Biology, Chemistry and Medicine. M. B. Sporn, A. B. Roberts, and D. S. Goodman, editors. Raven Press, New York. 387–441.

27. Humes, H. D., and D. A. Cieslinski. 1992. Interaction between growth factors and retinoic acid in the induction of kidney tubulogenesis in tissue culture. *Exp. Cell Res.* 201:8–15.

28. Yamada, M., C. Moritoh, M. Kawaguchi, and T. Okigaki. 1989. Growth, morphology, function, and morphogenetic properties of rat renal glomerular epithelial cells in vitro: effects of retinyl acetate. *Eur. J. Cell Biol.* 49:252–258.