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ADP-ribosyl cyclase: an enzyme that cyclizes NAD^+ into a calcium-mobilizing metabolite

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Cyclic ADP-ribose (cADPR)[1] is a metabolite of NAD^+ that is as active as inositol trisphosphate (IP_3) in mobilizing intracellular Ca^{2+} in sea urchin eggs. The activity of the enzyme responsible for synthesizing cADPR is found not only in sea urchin eggs but also in various mammalian tissue extracts, suggesting that cADPR may be a general messenger for Ca^{2+} mobilization in cells. An aqueous soluble enzyme, thought to be an NADase, has been purified recently from the ovotestis of *Aplysia californica* (Hellmich and Strumwasser, 1991). This paper shows that the *Aplysia* enzyme catalyzes the conversion of NAD^+ to cADPR and nicotinamide. The *Aplysia* enzyme was purified by fractionating the soluble extract of *Aplysia* ovotestis on a Spectra/gel CM column. The purified enzyme appeared as a single band of ~29 000 Da on SDS-PAGE but could be further separated into multiple peaks by high-resolution, cation-exchange chromatography. All of the protein peaks had enzymatic activity, indicating that the enzyme had multiple forms differing by charge. Analysis of the reaction products of the enzyme by anion-exchange high-pressure liquid chromatography (HPLC) indicated no ADP-ribose was produced; instead, each mole of NAD^+ was converted to equimolar of cADPR and nicotinamide. The identification of the product as cADPR was further substantiated by proton NMR and also by its Ca^{2+}-mobilizing activity. Addition of the product to sea urchin egg homogenates induced Ca^{2+} release and desensitized the homogenate to authentic cADPR but not to IP_3. Microinjection of the product into sea urchin eggs elicited Ca^{2+} transients as well as the cortical exocytosis reaction. Therefore, by the criteria of HPLC, NMR, and calcium-mobilizing activity, the product was identical to cADPR. To distinguish the *Aplysia* enzyme from the conventional NADases that produce ADP-ribose, we propose to name it ADP-ribosyl cyclase.

Introduction

Mobilization of intracellular calcium is an important signaling pathway in cells. It is generally accepted that inositol trisphosphate (IP_3) is a second messenger for mediating the calcium signal. The fact that it may not be the only messenger for the process is suggested by the findings of calcium mobilization with no or minimal change in endogenous IP_3 (McCann *et al.*, 1989; Saluja *et al.*, 1989; Chow and Jondal, 1990; Matozaki *et al.*, 1990). In addition, several other metabolites, such as sphingosine derivatives, arachidonic acid, cyclic IP_3, and inositol tetrakisphosphate, have been reported to have calcium mobilizing activity of their own (Wilson *et al.*, 1985; Wolf *et al.*, 1986; Snyder *et al.*, 1988; Ghosh *et al.*, 1990).

We have shown previously that a metabolite of NAD^+ is as potent as IP_3 in releasing calcium from intracellular stores in sea urchin eggs (Clapper *et al.*, 1987; Dargie *et al.*, 1990). Structural determination showed that the NAD^+ metabolite is cyclized ADP-ribose and it was named cADPR (Lee *et al.*, 1989). The cADPR-sensitive Ca^{2+} stores in sea urchin egg homogenates overlapped substantially with the IP_3-sensitive stores, but the release mechanisms activated by the two are distinct (Dargie *et al.*, 1990). Specific binding of cADPR to the Ca^{2+}-storing microsomes of sea urchin egg has been demonstrated and the receptor indeed appeared to be different from the IP_3 receptor (Lee, 1991).

Recently, the endogenous levels of cADPR in various mammalian tissues have been determined indicating that cADPR is a naturally occurring metabolite in cells (Walseth *et al.*, 1990). These results suggest that cADPR could be a general second messenger for mobilizing intracellular Ca^{2+} stores. Although the activity of the enzyme responsible for cyclizing NAD^+ to cADPR has been detected in a variety of mammalian tissue extracts as well as in sea urchin eggs (Rusinko and Lee, 1989), the enzyme from

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[1] Abbreviations: cADPR, cyclic ADP-ribose; HPLC, high-pressure liquid chromatography; IP_3, inositol trisphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

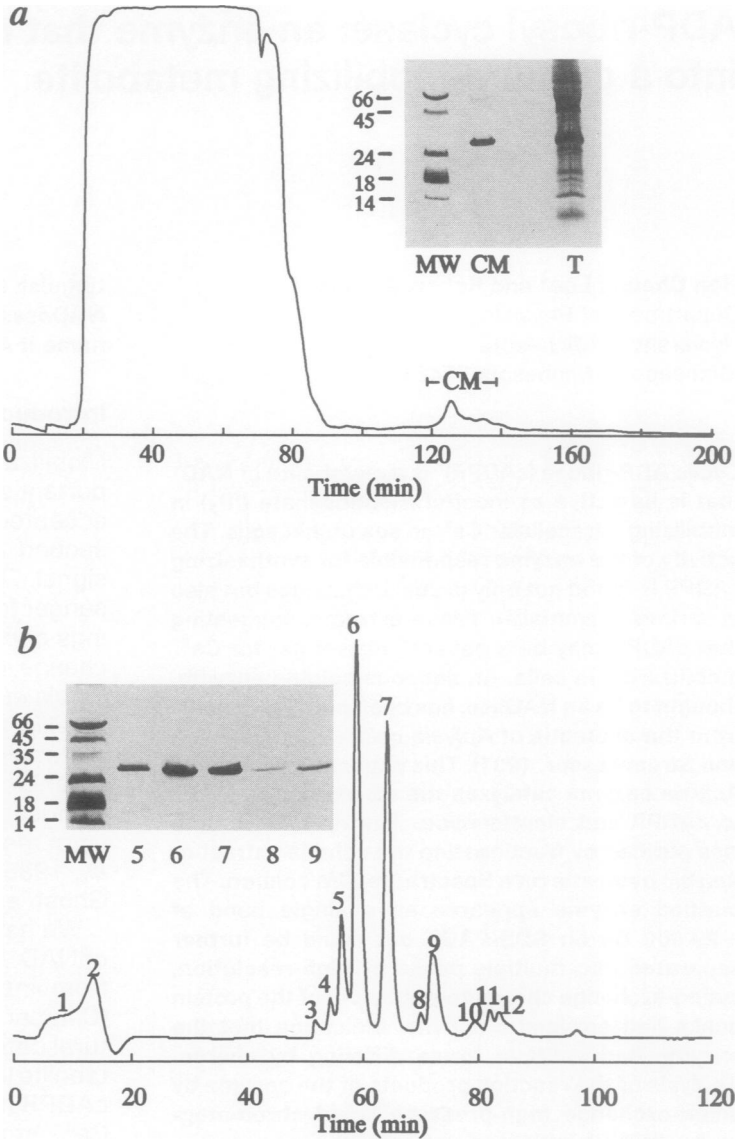

Figure 1. **Purification of the enzyme that catalyzes the conversion of NAD^+ to cADPR.** (a) The soluble extract of *Aplysia* ovotestis was chromatographed on a Spectra/Gel CM column. The NaCl gradient started at 80 min and reached 0.5 M NaCl at 200 min. The elution of proteins from the column was monitored by absorbance at 280 nm. Fractions with cADPR-producing activity were labeled CM. The inset shows a SDS-PAGE gel of the pooled and concentrated active fractions (CM), the total soluble extract (T), and molecular weight standards (MW). (b) The purified enzyme was analyzed with a high-resolution, cation-exchange column. Proteins were eluted with a NaCl gradient that started at 20 min and reached 0.35 M at 100 min. The inset shows a SDS-PAGE gel of the peaks labeled 5–9 and molecular weight standards (MW).

these sources has not been purified. On the basis of NAD^+ utilizing activity, an aqueous soluble enzyme has been purified recently from the ovotestis of *Aplysia californica* by Hellmich and Strumwasser (1991) and made available to us. This paper shows that the *Aplysia* enzyme catalyzes the conversion of NAD^+ to nicotinamide and a product that, by the criteria of HPLC, NMR, and calcium-mobilizing activity, is identical to cADPR. To distinguish this enzyme from the conventional NADases that produce ADP-ribose, we propose to name it ADP-ribosyl cyclase.

Results

The original purification method for the *Aplysia* NAD^+-utilizing enzyme employed three chromatography steps, with the first step being a cation-exchange column (Hellmich and Strumwasser, 1991). Figure 1a shows that a single Spectra/gel CM column could be used effectively for purification. The majority of proteins in the soluble extract of the ovotestis did not bind to the column at pH 8.0. The bound protein was eluted by a linear gradient of NaCl. Fractions were collected and assayed for production of cADPR with the use of either the calcium release assay or high-pressure liquid chromatography (HPLC) as detailed below. Only fractions eluted between 160 and 250 mM NaCl were active. When analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the pooled and concentrated fractions

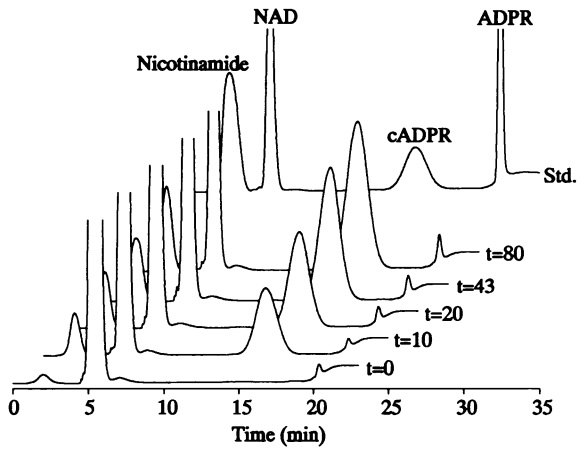

Figure 2. **Analysis of the reaction products of the *Aplysia* ADP-ribosyl cyclase by anion-exchange HPLC.** Chromatograms shown are of NAD^+ 2.5 mM before (t = 0) and at various times (t = 10, 20, 43, and 80 min) after incubating with the ADP-ribosyl cyclase at room temperature (23–25°C). Also shown is a chromatogram of four standards (std): nicotinamide, NAD^+, cADPR, and ADP-ribose (ADPR).

showed mainly a single band of ~29 000 Da. In a typical preparation starting with 118 mg of soluble extract from 3.6 g of tissue, 0.33 mg of purified enzyme was obtained. The specific activity of the enzyme increased from 21 μmol cADPR · mg^{-1} · min^{-1} in the extract to 1161 μmol cADPR · mg^{-1} · min^{-1} (assayed with 3 mM NAD^+ at pH 8.0 and 23°C), which was slightly higher than the enzyme purified by the original procedure (Hellmich and Strumwasser, 1991) and assayed by nicotinamide producing activity. The recovery of the enzymatic activity was ~14%.

The *Aplysia* NADase has been shown to occur in multiple isoforms by two-dimensional gel electrophoresis and immunoblotting (Hellmich and Strumwasser, 1991). The purified enzyme was further analyzed with a high-resolution, cation-exchange column, and the result is shown in Figure 1b. Multiple peaks were eluted and were collected individually. All were found to be enzymatically active except peaks 1, 2, and 10. On SDS-PAGE, the major peaks (5–9) all showed a single 29 000-Da band. It thus appears that the enzyme exists in multiple forms differing by charge.

The reaction products of the enzyme were analyzed by anion exchange HPLC. The last chromatogram in Figure 2 shows the elution time of four standards: nicotinamide, NAD^+, cADPR, and ADP-ribose. The chromatogram of the substrate NAD^+ before (t = 0) incubating with the enzyme showed mainly a single peak with contaminating amounts of nicotinamide and ADP-ribose, which were breakdown products of NAD^+. Incubation of NAD^+ with the enzyme produced progressive increases in both cADPR and nicotinamide, with very little, if any, change in ADP-ribose. It is clear that the enzyme was not an NADase, because it did not produce ADP-ribose. Figure 3 shows a time course of changes of substrate and products. The decrease in NAD^+ could be quantitatively accounted for by equimolar increase in cADPR and nicotinamide. The reaction appeared to slow down with time, which could be due either to product inhibition or to inactivation of the enzyme with prolonged incubation. The enzyme, therefore, cyclized one molecule of NAD^+ into a molecule of cADPR with the release of a molecule of nicotinamide. To distinguish this enzyme from the conventional NADase, we propose to name it ADP-ribosyl cyclase.

To further substantiate that the reaction product was indeed cADPR, the corresponding peak from the HPLC was collected and analyzed by NMR. Figure 4 shows the proton NMR spectrum of the reaction product. Only two peaks are present in the region between 8 and 9.2 ppm, which corresponded to the two adenine protons (H_A8 and H_A2). All protons of the nicotinamide ring were absent. The two doublets at around 6.2 ppm (H1′ and H_A1') and the triplet at 5.45 ppm (H_A2') are characteristic of cADPR and represent, respectively, the two anomeric protons and the number two proton of the ribose unit linked to the adenine group. The spectrum was, in fact, identical to cADPR gen-

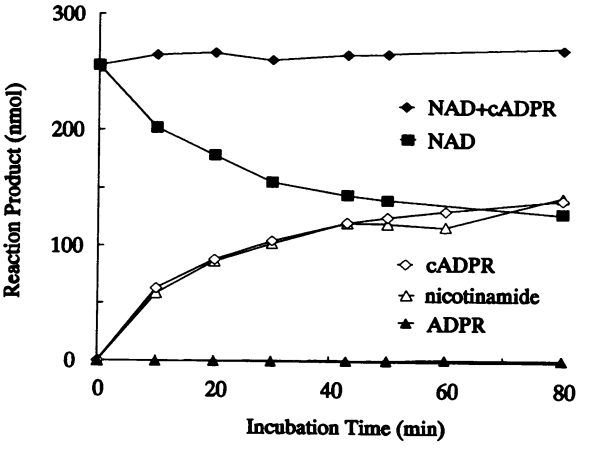

Figure 3. **Time course of changes in reactant and products during the *Aplysia* ADP-ribosyl cyclase enzymatic reaction.** The amounts of reactant and products present in the mixture at various times after the incubation were determined by comparison of the integrated peak areas from the HPLC chromatograms of Figure 2 with standards.

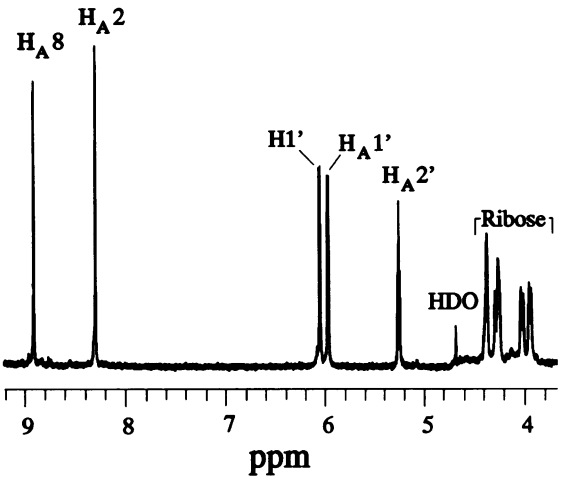

***Figure 4.* A proton NMR spectrum of the reaction product of the *Aplysia* ADP-ribosyl cyclase.** The reaction product was purified by HPLC and dissolved in D_2O. The intensity of the HDO peak was suppressed by presaturation.

erated by extracts from rabbit livers (Rusinko and Lee, 1989) and sea urchin eggs (Lee *et al.*, 1989).

The calcium-mobilizing activity of the reaction product is shown in Figure 5. At various times during the enzyme reaction, a small aliquot of the mixture was added to sea urchin egg homogenate. Calcium release from the homogenate was monitored by the calcium indicator Fluo 3. Figure 5a shows that the calcium release activity progressively increased during the reaction, reflecting the production of cADPR. A characteristic of the egg homogenate is that the calcium release activity can be desensitized to large dose of cADPR (Clapper *et al.*, 1987; Dargie *et al.*, 1990). Figure 5b shows that addition of a large dose of the HPLC-purified reaction product elicited a large calcium response. After this the homogenate was then allowed to resequester the released calcium. Subsequent addition of authentic cADPR did not produce a second calcium release. The homogenate, however, was still responsive to IP_3. Similarly, if the homogenate was desensitized first to cADPR then it became insensitive to the reaction product, as shown also in Figure 5b. This cross-desensitization behavior indicated that the *Aplysia* enzyme reaction product was functionally identical to cADPR.

Figure 6 shows microinjection of cADPR produced by the *Aplysia* ADP-ribosyl cyclase into a sea urchin egg induced a large calcium transient, which was measured microfluorimetrically with Indo 1 as the calcium indicator. The indo 1 fluorescence ratio increased from 0.27 to 0.74.

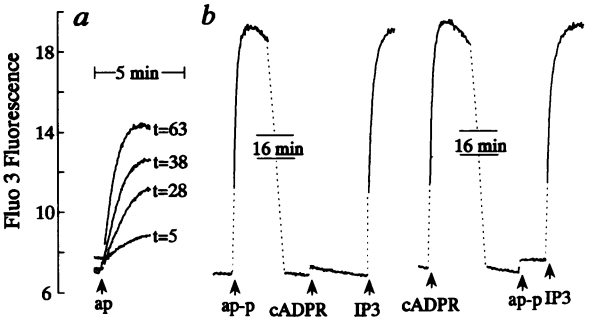

***Figure 5.* Calcium mobilizing activity of the reaction product of the *Aplysia* ADP-ribosyl cyclase.** (a) Progressive increase in calcium release activity after incubation of NAD^+ with the ADP-ribosyl cyclase. NAD^+ was incubated as described in Figure 2. At the arrow labeled ap, 1 μl of the 500-fold-diluted incubation mixture was added to 0.2 ml of sea urchin egg homogenate, and the resultant calcium release was monitored by the calcium indicator Fluo 3. The time (t) of incubation (in minutes) is listed beside each calcium release curve. (b) Desensitization of the egg homogenate to cADPR induced by the reaction product. A large dose (1.8 μM) of the HPLC-purified reaction product (ap-p) was added to the egg homogenate to elicit a large calcium response. The homogenate was allowed to resequester the released calcium, which took ~16 min. Subsequent addition of authentic cADPR (1.5 μM) did not produce any calcium release. The homogenate, however, was still sensitive to inositol trisphosphate (IP_3, 1.5 μM). Similar results were obtained when the order of addition of ap-p and cADPR was reversed.

Calibration with calcium-ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (Ca-EGTA) buffers indicated that ratio values of 0.2 and 0.8 corresponded to intracellular cal-

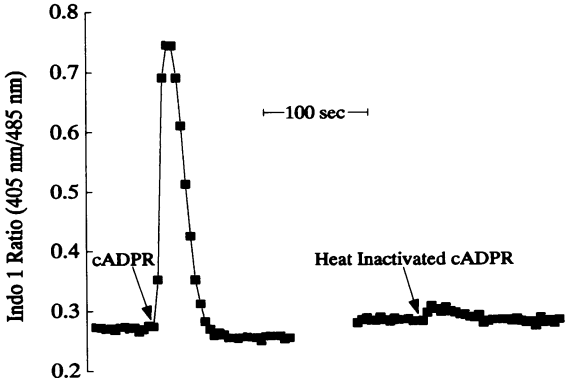

***Figure 6.* Changes in intracellular calcium concentration induced by microinjection of *Aplysia* cADPR.** A *Lytechinus pictus* egg was preloaded by microinjection with the calcium indicator Indo 1. *Aplysia* cADPR was microinjected into the egg at the time indicated by the arrow. A rapid calcium transient was induced as indicated by the increase in the fluorescence intensity ratio of Indo 1. The same cADPR was heated for 30 min in boiling water to inactivate its biological activity. Microinjection of the heat-inactivated cADPR did not produce any calcium change.

cium concentrations of ~0.2 and ~1 μM, respectively. The injection volume was ~0.7% of the cell volume, and the concentration of cADPR in the micropipette was 21 μM. Assuming uniform distribution, the intracellular concentration of cADPR should be ~0.15 μM. The injected egg also underwent a cortical exocytosis reaction, resulting in formation of the fertilization envelope (not shown). Heat treatment has been shown previously to break down cADPR and inactivate its calcium release activity (Dargie *et al.*, 1990). As a control, heat-inactivated *Aplysia* cADPR was microinjected into another egg and no calcium response was seen. Of the 10 eggs microinjected with *Aplysia* cADPR, 9 underwent cortical exocytosis, indicating intracellular calcium release. None of the 10 control eggs injected with heat-inactivated *Aplysia* cADPR underwent cortical reaction. These results show that the *Aplysia* cADPR can mobilize calcium in intact sea urchin eggs as well as in the cell-free homogenate system.

The substrate specificity of the *Aplysia* ADP-ribosyl cyclase was investigated by incubating the enzyme with 1 mM of various NAD^+ analogues at pH 7.0 for 1 min and assayed for production of cADPR by the use of the calcium release assay. Analogues tested were α- and β-NAD^+, ADP-ribose, NADH, NADP, NADPH, nicotinamide mononucleotide, deamino NAD^+, and nicotinic acid adenine dinucleotide. Only NADH could partially substitute for β-NAD^+. The ADP-ribosyl cyclase, therefore, showed a high degree of substrate specificity. ADP-ribose was not a substrate, indicating that the enzyme directly cyclized NAD^+ into cADPR instead of going through ADP-ribose as an intermediate step. This is consistent with the results of Figures 2 and 3, which show no increase in ADP-ribose during the reaction. The reaction was stereospecific because α-NAD^+ was not a substitute. The stereospecificity suggested the possibility that the adenyl-NH_2 group attacks the anomeric carbon of the terminal ribose from the α-direction and displaces the nicotinamide group.

Discussion

We have previously shown that the ADP-ribosyl cyclase is an ubiquitous enzyme (Rusinko and Lee, 1989). Its activity can be detected in a variety of mammalian tissue extracts as well as invertebrate tissues such as sea urchin eggs. The purified *Aplysia* ADP-ribosyl cyclase (Hellmich and Strumwasser, 1991) characterized in this study shares with the mammalian ADP-ribosyl cyclase the same high degree of substrate specificity (Rusinko and Lee, 1989). The physical properties of the two enzymes, however, appear to be quite different. The mammalian enzyme has been partially characterized recently (Lee, 1991). It is a membrane-bound enzyme. Its activity cannot be extracted from the membranes by high salt treatment but can be solubilized by detergent. It binds readily to anion exchange columns such as DEAE cellulose at around neutral pH, indicating that it is anionic. This is in contrast to the cationic nature of the *Aplysia* ADP-ribosyl cyclase. The mammalian enzyme is also quite large because it cannot pass through 30K cutoff filters even when solubilized. On a gel filtration column, the solubilized enzyme is eluted close to alcohol dehydrogenase, a protein of 150 000 Da. It thus appears that the membrane-bound form of the ADP-ribosyl cyclase found in various mammalian tissues is very much larger than the soluble form found in *Aplysia* ovotestis. However, the two forms of the enzyme may be related and could represent post-synthetic modifications. It is possible that the functional enzyme in mammalian cells is first synthesized as a small soluble subunit similar to the *Aplysia* enzyme and later linked to membrane components. Indeed, the ADP-ribosyl cyclase inside cells must be tightly regulated to prevent depletion of cellular NAD^+. It is intriguing to think that the association of the ADP-ribosyl cyclase with membranes may be a means for providing the necessary regulatory mechanisms.

NAD^+ glycohydrolase (EC 3.2.2.5), commonly known as NADase, is classified as an enzyme that hydrolyzes the ribose-nicotinamide linkage of NAD^+ to produce nicotinamide and ADP-ribose. Our results showed that the *Aplysia* enzyme does not produce any ADP-ribose, but instead cyclizes quantitatively 1 mole of NAD^+ to 1 mole of cADPR with the release of 1 mole of nicotinamide. It is clear that the *Aplysia* enzyme is not an NADase but the NAD^+-cyclizing activity we have described previously (Rusinko and Lee, 1989; Lee, 1991). Continued reference to the enzyme as NADase would be likely to create unnecessary confusion. Because the enzyme cyclizes NAD^+ into cyclic ADP-ribose, we therefore propose to name it ADP-ribosyl cyclase. One of the products of NADase, ADP-ribose, is relatively difficult to assay and many studies on NADase rely, instead, on following the disappearance of NAD^+ or the production of nicotinamide. These two assays, of course, cannot distinguish between an NADase and an ADP-ribosyl cyclase. Furthermore, because

cADPR is a recent discovery and because it is quite similar to ADP-ribose, some of the older assays for ADP-ribose may not be able to distinguish between the two. In the light of the present study and our previous demonstration of widespread occurrence of the ADP-ribosyl cyclase activity, some of the previous studies on NADases may require reexamination. A similar conclusion has been reached by Hellmich and Strumwasser (1991).

Methods

Purification of the ADP-ribosyl cyclase

Soluble extracts of *Aplysia* ovotestis were prepared by powdering the frozen tissue in liquid N_2 and homogenizing in buffer A (250 mM sucrose, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) 1 mM dithiothreitol, 1 mM EDTA, pH 8.0) containing 2.5 μg/ml soybean trypsin inhibitor, 3 μg/ml leupeptin, and 1 μg/ml aprotinin, as described previously (Hellmich and Strumwasser, 1991). The extract was centrifuged for 1 h in a Ti50.2 rotor (Beckman, Fullerton, CA) at 40 000 rpm (0–5°C) and the supernatant was loaded onto a Spectra/Gel CM column (Spectrum, Los Angeles, CA). The bound proteins were eluted with a linear gradient of 0–0.5 M prepared in buffer A without sucrose. The cADPR producing activity was assayed by incubating 1 μl of each fraction with 0.1 ml of 1 mM NAD^+ (pH 7.5) for 20 min at room temperature, and the reaction products were analyzed by HPLC as described below. Active fractions were pooled and concentrated with Centriprep 10 filters (Amicon, Danvers, MA) and loaded onto a SP-5PW column (Waters Associates, Milford, MA). Protein peaks eluted by a linear gradient of 0–0.35 M NaCl prepared in buffer A without sucrose were collected individually. The cADPR-producing activity was assayed by incubating 1 μl of each fraction with 0.1 ml of 1 mM NAD^+ (pH 8.0) for 1 min at room temperature. The mixture was diluted 10-fold and its calcium release activity was assayed by adding 1 μl to 0.2 ml of sea urchin egg homogenate as described below.

Analysis of the reaction products by HPLC

The purified *Aplysia* ADP-ribosyl cyclase (0.2 μg) was added to 5 ml of 2.5 mM NAD^+ at pH 7.0. Aliquots (0.1 ml) of the mixture were removed before and at various times after addition of the enzyme and quick frozen in acetone-dry ice mixture for later analysis. The samples were analyzed by HPLC using a column packed with the gel AG MP-1 (BioRad, Richmond, CA) (Axelson *et al.* 1981; Lee *et al.*, 1989). The chromatograms were developed with a nonlinear gradient of trifluoroacetic acid starting at 0% B (solvent B was 150 mM trifluoroacetic acid in water and solvent A was water), step increased to 2% B at 1 min and held for 6 min, linearly increased to 4% B in 5 min, linearly increased to 8% B in 5 min, step increased to 100% and held for 6 min. The column was calibrated with four standards: nicotinamide, NAD^+, cADPR, and ADP-ribose. The amounts of reactant and products present in the mixture at various times after the incubation were determined by comparison of the integrated peak areas from the HPLC chromatograms with standard curves.

Calcium release assay

Sea urchin egg homogenates were used as a biological assay for calcium release activity. The homogenates were made from *Lytechinus pictus* eggs, stored frozen at −70°C, and diluted to 2.5% when needed (Dargie *et al.*, 1990). The measurement was performed in a microfluorimetric cuvette using Fluo 3 as the Ca^{2+} indicator. The temperature of the cuvette was maintained at 17°C by a circulating water bath, and the homogenate (0.2 ml) was continuously mixed with a magnetic stirring bar.

Preparation of authentic cADPR

Partially purified ADP-ribosyl cyclase was prepared from dog brain extracts as described previously (Lee, 1991). Dog brain ADP-ribosyl cyclase is a membrane-bound protein. Brain homogenates were fractionated by sucrose density centrifugation. Fractions at 12–47% sucrose interface were collected and incubated with 2 mM NAD^+ for 1–3 h at 37°C. After the incubation, the membranes were removed by centrifugation and the product, cADPR, in the supernatant was purified by preparative HPLC using an AG MP-1 column. The cADPR preparations were judged pure by HPLC and proton NMR. The concentration of the sample was determined by absorbance at 254 nm using the extinction coefficient of 14 300/M/cm determined previously (Lee *et al.*, 1989).

Other methods

cADPR was microinjected into *Lytechinus pictus* eggs by pressure as described previously (Dargie *et al.*, 1990). The injection volume was calibrated by measuring the size of the droplet ejected into a drop of silicon oil. Changes in intracellular Ca^{2+} concentration were monitored by digital microfluorimetric techniques using Indo 1 as the Ca^{2+} indicator as described previously (Dargie *et al.*, 1990). SDS-PAGE was performed and silver stained with the Phast-System (Pharmacia, Piscataway, NJ) using a 20% gel. Proton NMR measurement was performed with a 500-MHz spectrometer (General Electric) and samples were dissolved in D_2O.

Acknowledgments

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