Cyclic GMP-dependent and -independent Effects on the Synthesis of the Calcium Messengers Cyclic ADP-ribose and Nicotinic Acid Adenine Dinucleotide Phosphate

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Cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) have been shown to mobilize intracellular Ca\(^{2+}\) stores by totally independent mechanisms, which are pharmacologically distinct from that activated by inositol trisphosphate. Although cADPR and NAADP are structurally and functionally different, they can be synthesized by a single enzyme having ADP-ribosyl cyclase activity. In this study, three different assays were used to measure the metabolism of cADPR in sea urchin egg homogenates including a radioimmunoassay, a Ca\(^{2+}\) release assay, and a thin layer chromatographic assay. Soluble and membrane-bound ADP-ribosyl cyclases were identified and both catalyzed NAD to produce cADPR. The soluble cyclase was half-maximally stimulated by 5.3 μM cGMP, but not by cAMP, while the membrane-bound form was independent of cGMP. The two forms of the cyclase were also different in the pH dependence of utilizing nicotinamide guanine dinucleotide (NGD), a guanine analog of NAD, as substrate, indicating they are two separate enzymes. The stimulatory effect of cGMP required ATP or ATPγS (adenosine 5′-O-(3-thiotriphosphate)) and cGMP-dependent kinase activity was shown to be present in the soluble fraction. The degradation of cADPR to ADP-ribose was catalyzed by cADPR hydrolase, which was found to be predominantly associated with membranes. Similar to the membrane-bound cyclase, the cADPR hydrolase activity was also independent of cGMP. Both the soluble and membrane fractions also catalyzed the synthesis of NAADP through exchanging the nicotinamide group of NADP with nicotinic acid (NA). The base-exchange activity was independent of cGMP and the half-maximal concentrations of NADP and NA needed were about 0.2 mM and 10 mM, respectively. The exchange reaction showed a preference for acidic pH, contrasting with the neutral pH optimum of the cyclase activities. The complex metabolic pathways characterized in this study indicate that there may be a multitude of regulatory mechanisms for controlling the endogenous concentrations of cADPR and NAADP.

The sea urchin egg is a good model system for investigating mechanisms of Ca\(^{2+}\) mobilization. Studies utilizing this system have led to the discoveries of two novel Ca\(^{2+}\) messengers, cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) (1–4). The Ca\(^{2+}\) release mechanisms activated by them are distinct and both are independent from that activated by inositol trisphosphate (IP3) (1, 4, 5). Cells responsive to cADPR have now been shown to be widespread and include those from protozoa (6), plant (7), invertebrate (1), and amphibian (8) as well as from a variety of mammalian species (Ref. 9; reviewed in Refs. 10–12). In the case of sea urchin eggs, evidence indicates cADPR, in conjunction with IP3, is responsible for mediating the Ca\(^{2+}\) mobilization associated with fertilization. Thus, inhibition of either the cADPR or the IP3 receptor alone is not sufficient to inhibit fertilization, but the blockage of both receptors by specific antagonists inhibits the Ca\(^{2+}\) mobilization (13–15). A unique feature of the cADPR pathway is that it can also be activated by an exogenous agonist, nitric oxide, a gaseous messenger, via elevation of cGMP concentration (16–18).

Cyclic ADP-ribose is synthesized from NAD by a ubiquitous enzyme, ADP-ribosyl cyclase (Refs. 19 and 20; reviewed in Refs. 21 and 22). In addition to cyclizing NAD, the cyclase can also catalyze the exchange of the nicotinamide group of NADP with nicotinic acid (NA) to produce NAADP (23). It is thus an important enzyme in Ca\(^{2+}\) signaling. Two types of cyclase have been characterized. A soluble form is abundant in Aplysia ovotestis and its crystal structure has recently been solved (24). A membrane-bound homolog of the Aplysia cyclase is CD38 (25, 26), which is also a lymphocyte differentiation antigen (reviewed in Ref. 27). However, neither of the these two enzymes has been shown to be regulated by cGMP. The egg cyclase, although having an activity among the lowest of all tissue extracts measured so far (20), is particularly important because of its cGMP dependence, a typical characteristic of a signaling enzyme. In this study, three different assays with sensitivity suitable for measuring the low cyclase activity in egg extracts were developed and used. Results show the presence of two types of cyclase and that the soluble form is sensitive to cGMP. A degradation activity hydrolyzing cADPR to ADP-ribose is present predominantly in the membranes. In contrast, both the soluble and membrane fractions can also catalyze the synthesis of NAADP via a base-exchange reaction. The egg extract is thus an important source for characterizing and purifying various

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* The abbreviations used are: cADPR, cyclic ADP-ribose; ATPγS, adenosine 5′-O-(3-thiotriphosphate); cGDP, cyclic GDP-ribose; IP3, inositol trisphosphate; NA, nicotinic acid; NAADP, nicotinic acid adenine dinucleotide phosphate; NAD, nicotinamide adenine dinucleotide; NGD, nicotinamide guanine dinucleotide; RIA, radioimmunoassay; HPLC, high performance liquid chromatography.
components of the complex pathways involved in the metabolism of the Ca^{2+} messengers, cADPR and NAADP.

MATERIALS AND METHODS

Fractionation of Egg Homogenates—Homogenates were prepared from sea urchin egg as described previously and stored at ~80 °C (28). Fractions were prepared from freshly thawed (20 min at 17 °C) homogenates by centrifugation for 30 min at 430,000 × g. The supernatants were further filtered with a 0.2-μm filter. The membrane pellets were resuspended in buffer A, described below, containing the ATP regenerating system.

Fractionation was also performed by Percoll density gradient centrifugation (29). Homogenates (2 ml) were layered on top of 10 ml of 25% Percoll in buffer A. After 30 min of centrifugation at 27,000 × g (10 °C), the bottom of the centrifuge tube was punctured and 10 fractions of equal volume were collected.

Preparation of Egg Homogenates for the Ca^{2+} Release Assay—Egg homogenate was thawed at 17 °C and diluted in steps from 25% to 12.5% in 250 mM potassium glutonate, 250 mM N-methyglycine, 1 mM MgCl₂, 20 mM Hepes, pH 7.2 (buffer A) containing 0.5 mM ATP, 8 mM phosphocreatine, 2 units/ml creatine kinase, and 3 μM fluo-3. For the assay of ADP-ribosyl cyclase activity, the diluted homogenate was supplemented with 10 mM nicotinamide and 1 mM caffeine to inhibit conversion of transferred NAD to cADPR (30) during the Ca^{2+} release process (31), respectively.

HPLC—A standard anion exchange column (15 × 0.7 cm) packed with AG MP-1 (Bio-Rad) and gradient elution with trifluoroacetic acid were used to analyze nucleotides in these experiments under conditions described previously (32). The gradient of trifluoroacetic acid used started at 0% B (solvent B is 150 mM trifluoroacetic acid in water, solvent A is water) and was held at 0% for 1 min, increased linearly from 4% to 1 from 6 to 11 min, increased linearly to 8% from 6 to 11 min, increased linearly to 16% from 11 to 13 min, and held at 100% until 17 min. The column was calibrated with nicotinamide, NAD, cADPR, ADP-ribose, ADP, ATP, NGD, cGMP, and GDP-ribose. cADPR and cGMP are released by incubation of NAADP and NGD, respectively, with the ADP-ribosyl cyclase from Aplysia californica as described previously and purified by HPLC (32).

Preparation of [32P]NAD and [32P]ADPR—[32P]NAD was prepared from [α-32P]ATP as follows: [α-32P]ATP (1 mCi) was incubated with 10 μM β-nicotinamide mononucleotide, 10 mM MgCl₂, 5 mM dithiothreitol, 10 mM creatine phosphate, 50 μg/ml creatine kinase, 0.13 units/ml inorganic pyrophosphatase, and 0.1 units/ml alkaline phosphatase (12 units/ml), nucleotide pyrophosphatase (0.4 unit/ml), and apyrase (1.2 units/ml). None of these enzymes degrades cADPR (34). The samples were incubated with the enzymes at 37 °C for 4 h. The binding assay contained a dilution of the sample, [32P]cADPR, and cADPR antibody (1:10) in 100 mM HEPES, pH 7.5. After 1 h at 23 °C, the antibody-cADPR complex was precipitated with 13% polyethylene glycol and filtered with GF/C filters. The filters were washed three times with 2 ml of buffer containing 20 mM HEPES, pH 7.5, and 15% polyethylene glycol. A standard curve was constructed with concentrations of cADPR ranging from 0.1 to 100 nM cADPR, and the midpoint usually at about 2 nM cADPR.

Thin Layer Chromatography (TLC) Assay—The cyclase reaction was started by adding [32P]NAD (100,000 cpm/μl) to egg extracts and incubated for 0–90 min at 17 °C. At various times, a 10-μl aliquot of the reaction mixture was quenched with a 10-μl mixture containing a combination of hydrolytic enzymes as described above for the RIA. After 60 min of treatment with the enzymes at 37 °C, 2 μl was spotted on polyethyleneimine-cellulose and the plate was developed with a solvent containing 0.2 M NaCl and 30% ethanol. Authentic [32P]cADPR and [32P]NAADP were also spotted on the same plate for identifying the elution positions. The spots corresponding to [32P]cADPR were visualized by a phosphorimager and cut. The radioactivity of the cut spots was quantified by scintillation counting. To further ascertain that the spots corresponded to cADPR, selected duplicate samples were additionally treated with CD38 (300 μg/ml) for 60 min at 37 °C to specifically hydrolyze cADPR to ADP-ribose. The latter migrated near the origin (R₀ = 0.06) well separated from cADPR (R₀ = 0.44) and NAADP (R₀ = 0.66).

Measurement of the Synthesis of NAADP by the Base-exchange Reaction—Egg extracts were incubated with 50 mM nicotinic acid at pH 5, adjusted with acetic acid. The reaction was quenched with 100 mM HCl. Samples were centrifuged in a microcentrifuge for 15 min to remove the precipitated proteins. The supernatants were neutralized with Tris base and incubated with 5 units/ml apyrase with 2 mM MgCl₂ for 30 min at 20–23 °C to remove ATP and ADP. Afterward, the samples were diluted 20-fold with 0.05% SDS and analyzed by HPLC as described above. Known standards were used to calibrate the area of the NAADP peak (23).

Ca^{2+} Release Assay—The base-exchange reaction was started and quenched as described above for the HPLC assay. The HCl-quenched samples were stable in acid at 4 °C, and 2–4-μl aliquots were added directly to the egg homogenates for determining the Ca^{2+} releasing activity, which was calibrated with authentic NAADP.

cGMP-dependent Kinase Assay—A specific cGMP-sensitive kinase assay was developed by using conditions that inhibit the cAMP-dependent kinase and the Ca/calmodulin kinase activities. The reaction mixture contained 10 μg/μl cGMP-sensitive protein kinase inhibitor (rabbit sequence), Kemptide, 10 mM MgCl₂, 50 μg/ml cAMP-dependent protein kinase inhibitor, 10 mM NaF, 50 μM β-glycerophosphate, 1 mM EGTA, 1 mM dithiothreitol, and 20 mM Tris, pH 7.5. Prior to the kinase assay the supernatant of sea urchin homogenate was concentrated 4-fold with Centricon 100 filters by centrifugation for 15 h at 2500 rpm. The concentrates (0.2–0.3 ml) were desalted by a 0.7 × 11-cm column filled with ACA 202 (LKB) gel, and 1-ml fractions (about 0.15 μl) were collected. The protein peak (usually fraction 20) was completely removed from the salt peak (fraction 35), which was measured with a refractometer. The pooled protein peak was diluted to about 1 mg/ml for the kinase assay. Reactions were stopped by spotting 25 μl of the reaction on a P-81 filter and immersing the filter in 200 mM phosphoric acid (35). The filters were washed four times in 200 mM phosphoric acid and counted for 32P.

Other Materials—The catalytic domain of human CD38 was expressed and produced in yeast as a secretory protein as described previously (36). NAD⁺, NGD⁺, ATP, ATP-S, CAMP-dependent protein kinase inhibitor (rabbit sequence), Kemptide, β-glycerophosphate, bovine serum albumin, Porell, nucleotide pyrophosphatase, NADase (from Neurospora crassa), apyrase, alkaline phosphatase, and trypsin were obtained from Sigma. [α-32P]ATP and [γ-32P]ATP were purchased from NEN Life Science Products. Mammalian cGMP-dependent protein kinase was purchased from Calbiochem. P-81 filters were from Whatman. Centricon filter units were from Amicon. Protein content was measured with a Bio-Rad protein assay reagent, and bovine serum albumin was used as standard.

RESULTS

Metabolism of cADPR in Egg Extracts

Assays for cADPR—The existence of an enzyme that can cyclize NAD was first identified in sea urchin egg extracts, which led to the discovery of cADPR (1, 2). The ADP-ribosyl cyclase activity of the egg extracts is, however, among the

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ADP-ribose cyclase activity. Three independent assays, an RIA, a Ca\(^{2+}\) release assay, and a radioactive thin layer chromatography (TLC) assay, were used to measure the production of cADPR in egg supernatants. Details of the assays are described under “Materials and Methods.” In all cases, the reactions were started by the addition of 1 mM NAD. cGMP (100 \(\mu\)M) was added 10 min prior to NAD. Top panel, three assays show similar stimulation of the soluble cyclase by cGMP (100 \(\mu\)M). Lower panel, a phosphorimager record of the TLC assay. Each lane represents one time point of the reaction, which was initiated by the addition of \((^{32}\text{P})\text{NAD}\) (0') with (+cGMP) or without (control) 100 \(\mu\)M cGMP. The cyclase reaction was done at 17 °C. Duplicates of the 90-min time point (90') were treated with CD38 (300 \(\mu\)g/ml, 60 min at 37 °C) to specifically hydrolyze cADPR. The amounts of \((^{32}\text{P})\text{cADPR}\) produced were quantified by scintillation counting of each spot. Error bars represent S.D. of four to six determinations.

![Fig. 1](image1.png)

**Fig. 1. Assays for ADP-riboyl cyclase activity.** Three independent assays, an RIA, a Ca\(^{2+}\) release assay, and a radioactive thin layer chromatography (TLC) assay, were used to measure the production of cADPR in egg supernatants. Details of the assays are described under “Materials and Methods.” In all cases, the reactions were started by the addition of 1 mM NAD. cGMP (100 \(\mu\)M) was added 10 min prior to NAD. Top panel, three assays show similar stimulation of the soluble cyclase by cGMP (100 \(\mu\)M). Lower panel, a phosphorimager record of the TLC assay. Each lane represents one time point of the reaction, which was initiated by the addition of \((^{32}\text{P})\text{NAD}\) (0') with (+cGMP) or without (control) 100 \(\mu\)M cGMP. The cyclase reaction was done at 17 °C. Duplicates of the 90-min time point (90') were treated with CD38 (300 \(\mu\)g/ml, 60 min at 37 °C) to specifically hydrolyze cADPR. The amounts of \((^{32}\text{P})\text{cADPR}\) produced were quantified by scintillation counting of each spot. Error bars represent S.D. of four to six determinations.

The homogenates were also assayed for cADPR hydrolyzing activity. The lower panel of Fig. 2 shows that, in contrast to the cyclase activity, cADPR hydrolyase was present essentially only in the membrane fraction and it also did not show any sensitivity to cGMP. The specific activity in the pellets was 65.1 ± 19 pmol/mg/min, which is about 7–8-fold higher than the cyclase activity. The egg homogenates thus possess a highly efficient pathway for removal of cADPR, consistent with it being a Ca\(^{2+}\) messenger.

The homogenates were also fractionated by Percoll gradient centrifugation. The main band of microsomes was in fraction 6 (Fig. 3) (29). Because the centrifugation force was relatively low (27,000 \(\times\) g for 30 min), the supernatant fraction (fraction 10)
contained soluble proteins as well as light membranes. The most remarkable feature is that the cyclase activity was found only in the light microsome and supernatant fractions, fraction 7 or higher (Fig. 3, top panel). The bottom of the gradient that contained mitochondria and yolk granules (29) had no detectable cyclase activity even though a high concentration of proteins was present (Fig. 3, bottom panel). When the cyclase activities were normalized with respect to protein concentration in each fraction, the specific cyclase activity was found to peak at fraction 7, a fraction just above the main microsomal band. In contrast, the distribution of the cADPR hydrolase was much more spread out (Fig. 3, middle panel), with the total hydrolase activity following mainly the protein distribution. After normalization, the specific hydrolase activity was centered around the middle of the gradient where the main band of microsomes was localized.

In addition to their difference in sensitivity to cGMP, the soluble and membrane forms of the cyclase also exhibit a dramatic difference in pH dependence of utilizing NGD, a guanine analog of NAD, as substrate. Fig. 4 shows that, at neutral pH, only the soluble cyclase can cyclize NGD to produce cGDPR with a specific activity of 110 ± 21 pmol/mg/min. A completely opposite result was found at acidic pH. Therefore, results on fractionation, cGMP sensitivity, and NGD utilization all suggest that there are two totally different forms of ADP-ribosyl cyclase in the eggs. The egg cyclases are very unusual in that their GDP-ribosyl cyclase activities are 10–15-fold higher than their ADP-ribosyl cyclase activities. In contrast, the Aplysia cyclase has much higher preference for using NAD than NGD as substrate (32, 39).

**Kinetic Parameters of the cGMP-dependent Cyclase**—The dependence of the soluble cyclase activity on cGMP concentration is shown in Fig. 5. The half-maximal effective concentration of 5.3 μM cGMP was calculated from a double-reciprocal plot of the data (inset). In a separate experiment, the dependence on NAD concentration was measured in the presence and absence of cGMP (Fig. 6). Activation by cGMP increased the $V_{max}$ value 3.8-fold from 9.3 to 38.8 pmol/mg/min. The $K_m$ value changed from 0.66 mM to 0.50 mM, only a 25% decrease. These results indicate that the stimulation by cGMP is primarily due to an increase in $V_{max}$ of the enzyme.

**The Stimulatory Effect of cGMP Requires ATP**—The egg homogenates were normally prepared in a medium containing 0.5 mM ATP. To determine if the cGMP-dependent stimulation of the egg cyclase requires ATP, apyrase was added to hydro-
lyze the ATP present. HPLC analyses confirmed complete removal of ATP in the samples (data not shown). The treated supernatants showed very low cyclase activity even in the presence of cGMP (Fig. 7). The sensitivity to cGMP can be restored with ATP, resulting in about 4-fold higher activity. ATPgS was even more effective, supporting about 10-fold higher cyclase activity. In contrast, the particulate cyclase activity showed no such dependence on ATP and was not affected by the apyrase treatment (data not shown).

The ATP requirement is consistent with the cGMP stimulation being mediated by protein phosphorylation, perhaps by a cGMP-dependent protein kinase. In general, proteins thiophosphorylated by ATPgS are more resistant to deactivation by protein phosphatases. This is the case for the soluble cyclase activated by cGMP in the presence of ATPgS. Egg supernatants assayed immediately after activation showed cyclase activity similar to that assayed 60 min later (Fig. 8). The rate of production of cADPR in either case was about 5-fold higher (13 pmol/mg/min) than control supernatants (2.5 pmol/mg/min) that had not been activated. These results show that, once the cyclase is activated by cGMP in the presence of ATPgS, it is stable for at least 60 min. In contrast, without the stabilization by ATPgS, the soluble cyclase can be inactivated by complete removal of ATP from the supernatants using a desalting column and the activity cannot be restored by subsequent addition of ATP and cGMP (data not shown).

Protein kinase G activity was indeed present in the eggs. As shown in Fig. 9, in the presence of cGMP, phosphorylation of Kemptide by the egg supernatant was 78% higher than control, whereas equimolar cAMP produced only about 30% stimulation. The protein kinase G activity was only a minor component since its detection required the presence of inhibitors for both protein kinase A (a peptide fragment of protein kinase A inhibitor) and the Ca2+-dependent protein kinase (EGTA), which together blocked 98% of the kinase activity in the supernatants. The egg protein kinase G, however, appeared to be quite unique since mammalian protein kinase G (Calbiochem) could not substitute for the egg enzyme for stimulation of soluble cyclase (data not shown).

**Metabolism of NAADP in Egg Extracts**

Assays for NAADP—The Aplysia ADP-ribosyl cyclase has been shown to be a multifunctional enzyme catalyzing not only the cyclization of NAD but also the exchange of the nicotinamide group of NADP with nicotinic acid (NA) to produce NAADP (23). Fig. 10 shows a time course of production of NAADP, which was measured by the Ca2+ release assay that the Ca2+ release was due to NAADP and not cADPR was verified by using 8-amino-cADPR as a specific antagonist of cADPR (33, 40) and by homologous desensitization, whereby prior treatment of egg homogenates with NAADP rendered them insensitive to subsequent challenge with NAADP but not...
Characteristics of the Synthesis of NAADP by the Base-exchange Reaction—Egg homogenates were fractionated and Fig. 11 shows that both the soluble and the particulate fractions could catalyze the synthesis of NAADP. The reaction catalyzed by the soluble fraction was not stimulated by cGMP, in contrast to the synthesis of cADPR. Additionally, the specific rates of synthesis of NAADP by the fractions were between 60 and 380 pmol/mg/min, much higher than the rate of synthesis of cADPR.

The dependence of the base-exchange reaction on the substrate concentration is shown in Fig. 12. The half-maximal value for NADP was about 0.2 mM and for NA was about 10 mM. The reaction shows a dramatic preference for acidic pH as shown in Fig. 13. The specific exchange rate at pH 5 was more than 9-fold higher than at neutral pH. Both the particulate (Fig. 13) and the soluble (data not shown) fractions show this unusual pH dependence, which is similar to that reported for the Aplysia cyclase (23). In contrast, the synthesis of cADPR by the particulate (Fig. 13) fraction shows no such preference for acidic pH; the optimum pH was at neutrality.

**DISCUSSION**

The sea urchin egg has been an ideal cellular model for investigating the mechanisms of Ca\(^{2+}\) mobilization both at the single-cell and biochemical levels. It was by using this model that the existence of Ca\(^{2+}\) release mechanisms other than that mediated by IP\(_3\) was first demonstrated (1, 2, 4, 5). Using caged analogs and microinjection, it was shown conclusively that both cADPR and NAADP can mobilize Ca\(^{2+}\) in live eggs (Refs. 42 and 43; reviewed in Ref. 44). That nitric oxide can activate the cADPR-mediated signaling pathway indicated that the synthesis of cADPR is regulated and that it can be activated by physiological stimuli (17). Because of the special role of sea urchin eggs in establishing cADPR as a Ca\(^{2+}\) messenger, it was of importance and interest to characterize the metabolism of cADPR and NAADP in this cell model.

Results in this study show that there are two forms of ADP-ribosyl cyclase in sea urchin egg, soluble and membrane-bound. This is established based on several criteria. First, the soluble cyclase cannot be sedimented by centrifugation by as high a force as 430,000 \(\times\) g for 30 min. Second, only the soluble cyclase shows stimulation by cGMP. Third, the cGMP-dependent stimulation of the soluble cyclase is critically dependent on ATP but

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**Fig. 9.** Presence of cGMP-dependent protein kinase activity in egg supernatants. Concentrated supernatants were fractionated, and the kinase activity was measured in the presence of a peptide inhibitor of protein kinase A as described under "Materials and Methods." Kemp tide was used as a substrate and its phosphorylation was determined in the absence (control) or presence of either 100 \(\mu\)M cGMP or 100 \(\mu\)M cAMP at 17 °C. The inset plots the specific protein kinase activities under the three conditions indicated. Error bars represent S.D. of triplicate determinations.

**Fig. 10.** Synthesis of NAADP via a base-exchange reaction. Egg supernatants were incubated with 1 mM NADP and 50 mM nicotinic acid at 23 °C. The production of NAADP was determined by the Ca\(^{2+}\) release assay as described under "Materials and Methods." In a separate experiment, samples incubated under similar conditions were analyzed by HPLC as described under "Materials and Methods." The inset shows the region of the chromatographs where authentic NAADP elutes. The area under the 120-min sample corresponds to 288 pmol of NAADP.

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with cADPR (4, 41). In addition to the Ca\(^{2+}\) release assay, NAADP production could also be directly measured by HPLC. The inset of Fig. 10 shows a series of HPLC chromatograms focusing on the elution region of authentic NAADP. That the chromatographic peaks were due to NAADP was verified by collecting the corresponding fractions and assaying for Ca\(^{2+}\) release activity.

**Characteristics of the Synthesis of NAADP by the Base-exchange Reaction—** Egg homogenates were fractionated and Fig.
The insensitivity of the membrane-bound cyclase to cGMP is not due to the lack of a suitable G-kinase in the membrane fraction since only marginal stimulation by cGMP is seen in the total homogenate, which can be accounted for by the stimulation of the soluble cyclase (cf. Fig. 2). Also, supplementing the membrane fraction with a supernatant fraction could not restore the cGMP stimulation (data not shown). The supernatant fraction contained G-kinase activity as determined by the kinase assay shown in Fig. 9, but its cyclase activity was inactivated by ATP removal using a desalting column as described under “Results.” Supplementing the membrane fraction with a recombinant form of the mammalian 1α isoform of cGMP-dependent protein kinase also failed to restore the cGMP sensitivity of the membrane cyclase (data not shown).

It is not unusual that there are two forms of ADP-ribosyl cyclase. The Aplysia cyclase is a soluble protein of about 30 kDa, whereas CD38 is a membrane-bound glycoprotein (reviewed in Refs. 21 and 22). Similarly, guanylyl cyclase is found as both soluble and particulate forms (45). What is unusual in the sea urchin egg is that the two forms of ADP-ribosyl cyclase are present in the same cell. The fact that the soluble cyclase is sensitive to cGMP makes it reasonable to assume that it is the signaling enzyme responsible for mediating the Ca2+-mobilizing effect of nitric oxide observed in sea urchin eggs (17). Thus the elevation of cGMP levels in eggs by nitric oxide (17) stimulates the soluble cyclase, resulting in increased production of cADPR and mobilization of the Ca2+ stores. In this scenario, cADPR is functioning as a Ca2+ messenger. Accumulating evidence suggests that cADPR can also function as a modulator, sensitizing the Ca2+-induced Ca2+ mechanism to Ca2+ (reviewed in Ref. 10). The membrane-bound cyclase could be responsible for regulating the local concentration of cADPR around the Ca2+ release channel and, by doing so, set the Ca2+ sensitivity of the Ca2+-induced Ca2+ release mechanism. Another finding reported in this study that is consistent with this proposal is the predominant association of the of cADPR hydrolyase with membranes. The presence of both synthesis and degradation activities would allow for fine adjustment of the local concentration of cADPR and thus the sensitivity of the Ca2+ release mechanism to Ca2+.

It is likely that the cGMP stimulation of the soluble cyclase is mediated by protein kinase G, which is consistent with previous results in intact egg and homogenates showing that the cGMP-activation of the cADPR-dependent pathway can be blocked by protein kinase inhibitors (16, 17). In this study, the cGMP stimulation of the soluble cyclase is found to require ATP and the presence of protein kinase G activity in the egg supernatant is demonstrated. The egg protein kinase G appears to be a minor component since its detection requires that at least 98% of the protein kinase A and the Ca2+-dependent protein kinase activities be blocked. The egg G kinase is nevertheless very unique. Unlike other forms of G-kinase, the G-kinase in the egg has a specific substrate, i.e. the soluble cyclase, and is not stimulated by equimolar cAMP. A recombinant form of the mammalian 1α isoform of cGMP-dependent protein kinase was also found to be unable to stimulate the soluble cyclase from eggs (data not shown), further demonstrating the specificity of the egg G kinase.

The egg extracts can also catalyze the synthesis of NAADP via a base-exchange reaction. The activity is present in both the soluble and membrane fractions, but no cGMP stimulation is observed in either fraction. It is known that the Aplysia cyclase can catalyze both the cyclization and base-exchange reactions (23). Whether the egg cyclase is also responsible for synthesizing NAADP remains to be shown. The specific exchange activity in the egg extracts, however, appears to be much higher.
than the cyclase activity. Similar to the Aplysia cyclase, the exchange activity catalyzed by the egg extracts also shows a remarkable preference for acidic pH as low as pH 5 (23). It is unlikely that, under physiological conditions, the cytoplasmic pH can be as acidic as pH 5. However, it should be noted that NAADP is present in the egg extracts, which should continue to signaling. In any case, it is clear that a synthetic pathway for optimum to produce sufficient amounts of NAADP for Ca\textsuperscript{2+} stores (4, 41). The exchange activity does not have to be at its pH can be as acidic as pH 5. However, it should be noted that exchange activity catalyzed by the egg extracts also shows a remarkable preference for acidic pH as low as pH 5 (23). It is unlikely that, under physiological conditions, the cytoplasmic release activator and only NAADP but also cADPR. In that regard, work is in progress to purify and characterize the soluble egg cyclase.

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