Differential regulation of nicotinic acid–adenine dinucleotide phosphate and cADP-ribose production by cAMP and cGMP

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The sea urchin egg has been used as a system to study calcium-release mechanisms induced by inositol 1,4,5-trisphosphate (IP₃), cADP-ribose (cADPR), and more recently, nicotinic acid–adenine dinucleotide phosphate (NAADP). In order that cADPR and NAADP may be established as endogenous messengers for calcium release, the existence of intracellular enzymes capable of metabolizing these molecules must be demonstrated. In addition, intracellular levels of cADPR and NAADP should be under the control of extracellular stimuli. It has been shown that cGMP

stimulates the synthesis of cADPR in the sea urchin egg. The present study shows that the sea urchin egg is capable of synthesizing and degrading NAADP. cADPR and NAADP synthetic activities appear to be separate, with different cellular localizations, pH and temperature optima. We suggest that in the sea urchin egg, cADPR and NAADP production may be differentially regulated by receptor-coupled second messengers, with cADPR production being regulated by cGMP and NAADP production modulated by cAMP.

INTRODUCTION

Calcium plays a major role in signal transduction, mediating important physiological events such as hormone secretion, muscle contraction and egg fertilization [1]. Cells have two major pathways to increase intracellular Ca²⁺ levels: by allowing Ca²⁺ influx across the plasma membrane, and by Ca²⁺ release from intracellular stores, including the endoplasmic reticulum [2].

The sea urchin egg has been used as a system to investigate calcium release from internal stores induced by inositol 1,4,5-trisphosphate (IP₃), cADP-ribose (cADPR), and more recently, nicotinic acid–adenine dinucleotide phosphate (NAADP) [3–5]. The mechanisms involved in these three Ca²⁺-release pathways have been studied in this system, including the channels gated by these molecules. IP₃ and cADPR act independently of one another on IP₃ and ryanodine receptors respectively, both of which reside on thapsigargin-sensitive endoplasmic reticular stores [3,4,6]. However, NAADP has been shown to release calcium from a thapsigargin-insensitive pool [7]; its mechanism of release has yet to be clearly defined [8], although the pharmacology of NAADP-induced Ca²⁺-release has been shown to be distinct from that of IP₃- and cADPR-induced Ca²⁺ release [9]. In addition, NAADP exhibits unique inactivation properties: the response to a concentration that would normally cause maximum Ca²⁺ release can be completely abolished by pre-treatment with concentrations of NAADP that are themselves below the threshold required to release calcium [10,11].

The Ca²⁺-mobilizing action of cADPR was first described in the sea urchin egg, and it has now been shown to release Ca²⁺ in a wide range of tissues, from plants through to several mammalian systems [6,12,13]. For example, cADPR has recently been shown to play a physiological role in cardiac contractility [14] and in catecholamine release from chromaffin cells [15]. cADPR is synthesized from β -NAD⁺ by an ADP-ribosyl cyclase enzyme and is degraded by cADPR hydrolase enzymes [16]. Two well-characterized enzymes, CD38 lymphocyte cell-surface antigen [17] and *Aplysia* ADP-ribosyl cyclase [18], have been shown to produce cADPR. CD38 is a bifunctional enzyme in that the same protein is capable of synthesizing and hydrolysing cADPR. The

Aplysia cyclase exhibits an extremely high cADPR-producing activity, but weak hydrolase activity. The *Aplysia* cyclase is localized within granules, whereas most CD38 is found extracellularly [17]. Such locations are, however, unsuited for a direct role in Ca²⁺ signalling. Neither of these proteins has been shown, unequivocally, to be involved in cADPR-induced Ca²⁺-release pathways. However, the sea urchin egg cyclase has been shown to be directly involved in cADPR-mediated Ca²⁺ release, playing a role in fertilization [19], and can be stimulated by cGMP [20]. cADPR production by CD38 and *Aplysia* ADP-ribosyl cyclase has not been demonstrated to be responsive to this second messenger, however, the cytoplasmic domain of CD38 has been reported to contain a consensus sequence for phosphorylation by cGMP-dependent protein kinases [21].

In addition to regulating cADPR metabolism, CD38 and *Aplysia* ADP-ribosyl cyclase are also able to use β -NAD⁺ as an alternative substrate, exchanging nicotinamide for nicotinic acid to produce NAADP, a recently identified calcium-mobilizing agent [22]. As the sea urchin egg enzymes that produce and degrade cADPR have been shown to be directly involved in Ca²⁺-signalling pathways, we have determined whether this system also possesses the enzymic machinery to metabolize NAADP and have investigated how NAADP metabolism may be regulated.

In this study we report that sea urchin egg subcellular fractions are able to synthesize and degrade NAADP and as for CD38 and *Aplysia* ADP-ribosyl cyclase, NAADP and cADPR synthesis show different pH dependencies. NAADP synthesis is potentiated by cAMP and has been localized to an entirely membrane-bound fraction. In contrast, the cADPR-producing enzyme is predominantly soluble and is 200-fold more sensitive to regulation by cGMP than by cAMP.

MATERIALS AND METHODS

Materials

Lytechinus pictus sea urchins were obtained from Marinus Inc. (Long Beach, CA, U.S.A.). Chemicals were supplied by Sigma,

Abbreviations used: cADPR, cADP-ribose; NAADP, nicotinic acid–adenine dinucleotide phosphate; IM, intracellular medium; IP₃, inositol 1,4,5-trisphosphate; IBMX, 3-isobutyl-1-methylxanthine; Rp-cAMPS, adenosine 3',5'-cyclic monophosphothioate, Rp isomer.

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except Fluo-3 (pentapotassium salt) and the Rp isomer of adenosine 3',5'-cyclic monophosphothioate (Rp-cAMPS), which were purchased from Molecular Probes and Calbiochem respectively.

Calcium-release assays

Homogenates of sea urchin eggs were prepared as described previously [4], and calcium-loading of intracellular stores was achieved by incubating for 3 h at 17 °C in an 'intracellular medium' (IM) consisting of 250 mM potassium gluconate, 250 mM *N*-methylglucamine, 20 mM Hepes and 1 mM MgCl₂, pH 7.2. Additions of 1 mM ATP, 10 units/ml creatine kinase and 10 mM phosphocreatine were made to achieve an ATP-regenerating system, plus 25 µg/ml leupeptin, 10 µg/ml aprotinin and 50 µg/ml soya bean trypsin inhibitor, as protease inhibitors, and 1 µg/ml oligomycin, 1 µg/ml antimycin and 1 mM sodium azide, as mitochondrial inhibitors. Fluo-3 (3 µM) was used as a Ca²⁺ indicator. Free Ca²⁺ was measured at 17 °C using 500 µl of homogenate in a Perkin-Elmer LS-50B fluorimeter at excitation and emission wavelengths of 490 nm and 535 nm respectively. Additions were made in 5 µl volumes and changes in relative fluorescence units were calibrated to known Ca²⁺ additions. cADP-ribose and NAADP concentration-response curves were constructed using standards in IM to estimate levels of cADPR or NAADP in samples from the enzyme assays.

Detection of cADPR and NAADP metabolism (discontinuous assay)

The ability of the sea urchin egg to synthesize cADPR was determined by incubating egg homogenate with β-NAD⁺ (2.5 mM, unless otherwise stated), and its ability to synthesize NAADP was determined by incubating egg homogenate with β-NADP⁺ (250 µM) and nicotinic acid (7 mM), unless otherwise stated. Synthesis of the calcium-releasing products was monitored over time, where 5 µl of reaction mixture was withdrawn and assayed immediately for activity that stimulates the ryanodine receptor or the NAADP-sensitive Ca²⁺ release mechanism, using the bioassay described above. The specificity of release was confirmed by desensitization to standard chemicals. Degradation of NAADP was assessed by monitoring the disappearance of 5 µM NAADP with time, using the bioassay. For all experiments using cGMP or cAMP, 100 µM 3-isobutyl-1-methylxanthine (IBMX) was added to the incubation to prevent the breakdown of cyclic nucleotides by phosphodiesterases.

Fractionation of sea urchin egg homogenates

L. pictus egg homogenates were fractionated by centrifugation for 60 min at 100 000 *g* at 4 °C. The supernatant was removed and retained as the 'soluble fraction' and the pellet was resuspended in IM, plus the ATP-regenerating system, as the 'membrane fraction'.

Protein concentrations were determined for these samples, and for all of the above samples tested in the enzyme assays, using the bichoninic acid protein assay (Sigma). All enzyme activity values, where expressed as mol/mg, are per mg of total protein.

Data analysis

Concentration-effect curves were fitted to data points by an iterative procedure using the Hill equation. From this EC₅₀ values were estimated (Kaleidograph software). Regression values (*R*²) were calculated for linear fits to plotted data using the

Microsoft Excel software package where *R*² = +1 for a straight line with positive slope. Statistical analysis was performed using Minitab by analysis of variance. *P* values were determined for statistical significance between sets of data using the modified Student's unpaired *t*-test [23].

RESULTS

Detection of NAADP metabolic activity in crude egg homogenates.

Figure 1(A) shows NAADP production with respect to time in sea urchin egg homogenates (12%, v/v) using exogenous β-NADP⁺ and nicotinic acid as substrates. The identity of the molecule produced in the incubation that was able to release Ca²⁺ was confirmed as NAADP, as shown in Figure 1(B). Pre-treatment of the homogenate with the incubation sample desensitized it to a subsequent addition of 500 nM NAADP, which alone causes maximal release of Ca²⁺. This experiment was also performed with a different sequence of additions, whereby an initial addition of authentic NAADP was made, which released Ca²⁺ maximally, followed by an incubation sample which was unable to cause Ca²⁺ release due to prior desensitization to NAADP. Subsequent additions of IP₃ and cADPR were made, but no desensitization to these responses was observed. Therefore

Figure 1 NAADP production in sea urchin egg homogenates

Egg homogenates (12.5%, v/v) were incubated with 250 µM β-NADP⁺ and 7 mM nicotinic acid, pH 5.0. (A) Increase in NAADP production with time; values are means ± S.E.M. for *n* ≥ 9. (B) Stores were allowed to re-sequester Ca²⁺ after the addition of an incubation sample and were subsequently re-challenged with a 500 nM NAADP standard, which alone elicits maximal Ca²⁺ release. Additions of cADPR and IP₃ were made in the same cuvette to demonstrate the lack of heterologous desensitization between the incubation sample and these agents.

Figure 2 Increasing substrate concentrations increases NAADP production

Egg homogenates (12.5%, v/v) were incubated with increasing concentrations of (A) β -NADP⁺ and (B) nicotinic acid. The corresponding NAADP production at 90 min, pH 7.0, is shown. Values are means $\pm$ S.E.M. for $n \geq 3$ –6.

the incubation sample was likely to have been releasing Ca²⁺ due to the presence of NAADP.

Increasing concentrations of β -NADP⁺ were applied in the presence of 7 mM nicotinic acid (Figure 2A). Maximal NAADP

Figure 4 Effect of temperature on NAADP and cADPR production

Egg homogenates (12.5%, v/v) were incubated either with 250 μ M β -NADP⁺ and 7 mM nicotinic acid for NAADP production, or with 2.5 mM β -NADP⁺ for cADPR production, pH 7.0. The temperature dependence of NAADP and cADPR production are shown (left and right y-axes respectively), where the shaded region * represents 'physiological temperature range' for the sea urchin egg.

Figure 5 NAADP degradation by sea urchin egg homogenates

Egg homogenates (12.5%) were incubated with 50 μ M NAADP. (A) Concentration of NAADP remaining versus time. (B) NAADP degraded (pmol per mg of total protein) with time. Values are means $\pm$ S.E.M. for $n \geq 3$.

Figure 3 Effect of pH on NAADP and cADPR production

Egg homogenates (12.5%, v/v) were incubated either with 250 μ M β -NADP⁺ and 7 mM nicotinic acid for NAADP production, or with 2.5 mM β -NADP⁺ for cADPR production. (A) Time course for NAADP production at pH 7.0 and pH 4.0. (B) NAADP and cADPR production at various pH levels. Values are means $\pm$ S.E.M. for $n \geq 3$.

production was achieved at 750 μ M β -NADP⁺. Increasing concentrations of nicotinic acid were added in the presence of 250 μ M β -NADP⁺ (Figure 2B), where maximal production was observed at 10 mM nicotinic acid. No NAADP was produced in the absence of either one of the two substrates. For further experiments studying the regulation of NAADP production, sub-maximal concentrations of substrates were used, i.e. 250 μ M β -NADP⁺ and 7 mM nicotinic acid respectively, so that the effect of any modulation could be clearly observed.

Production of NAADP was optimal at pH 5.0, as shown in Figure 3. This contrasts with the pH optimum for the β -NADP⁺ cyclization, as most cADPR was produced at pH 7.0. Figure 4 shows that the optimal temperature range for NAADP production was different from that for cADPR synthesis. NAADP synthesis was stable at 37 °C, whereas the ADP-ribosyl cyclase activity was reduced to approximately 50% of its maximum at this temperature. Between 15 and 19 °C, the physiological temperature range for the sea urchin egg, both NAADP and cADPR production approached an optimum level. NAADP production decreased at 60 °C and was abolished by heat treatment of the extract (results not shown), therefore this synthesis is likely to depend on an enzyme-catalysed reaction.

The sea urchin egg homogenate was also shown to be capable of degrading NAADP (Figure 5). The time taken to inactivate half of the 5 μ M NAADP added to the incubation mixture was approximately 60 min. However, no more than 75% of the 5 μ M NAADP added was metabolized after 3 h.

Figure 6 Localization of NAADP and cADPR production to soluble or membrane fractions

Soluble and membrane fractions of egg homogenates were tested for **(A)** NAADP production and **(B)** cADPR production, with respect to time. Values are means $\pm$ S.E.M. for $n \geq 3$.

The NAADP-producing activity was localized to the membrane fraction (Figure 6), so this enzyme is likely to be membrane associated. The NAADP-producing activity occurred at a location which was different from the site of 90% of the cADPR synthetic activity, which was in the soluble, cytosolic fraction.

Effect of cGMP and cAMP on NAADP production

Figure 7 shows that both cGMP and cAMP (500 μ M) were able to augment NAADP production in egg homogenates. The presence of IBMX alone, used to prevent cyclic nucleotide breakdown by phosphodiesterases, did not alter the production of NAADP. The increase in production appears to be greatest for the later time points. Incubation of cGMP or cAMP with homogenate in the absence of substrates for NAADP formation did not result in the production of a calcium-mobilizing agent (results not shown). The action of cAMP on the degradation of NAADP was investigated. Figure 7(D) shows that cAMP did not significantly alter the amount of NAADP degraded with time. This result was confirmed for other time points.

Figure 8 shows the concentration effect of cGMP and cAMP on both cADPR and NAADP production in egg homogenates. As previously shown [20,24], cGMP was able to effectively increase cADPR production in the sea urchin egg, above that of the control. The half-maximal effective concentration for cGMP on the cyclase activity was around 7 μ M. The extent of cADPR

Figure 7 Effect of cGMP and cAMP on NAADP production and degradation

All incubations were performed in the presence of 100 μ M IBMX, 250 μ M β -NADP $^{+}$ and 7 mM nicotinic acid. NAADP production was measured in the absence and in the presence of 500 μ M cGMP or 500 μ M cAMP: **(A)** typical Ca^{2+} -release traces at 90 min; **(B)** time courses for NAADP production. **(C)** Pooled data for the effect of 500 μ M cAMP ($n = 27$) and 500 μ M cGMP ($n = 12$) on NAADP production at 120 min. The effect of cAMP and cGMP was calculated to be highly significant for an increase in NAADP production over the control; **** $p < 0.0001$; *** $p < 0.001$, using an unpaired Student's t -test. **(D)** Effect of cAMP on degradation of NAADP at 120 min. Values are means $\pm$ S.E.M. for $n \geq 3$.

production was increased approximately 2-fold over the control by maximal concentrations of cGMP. cAMP had a much smaller effect in increasing cADPR production, with a half-maximal effective concentration of approximately 1.6 mM.

For NAADP production, however, the effects of cGMP and cAMP appeared to be quite different. cAMP was able to enhance NAADP production much more effectively than cGMP over the range of concentrations from 30 μ M and above. The enhancement of NAADP production due to cAMP does not appear to plateau, but continues to rise. At 3 mM cAMP, the production of NAADP was enhanced approximately 9-fold over the control, with an EC_{50} of around 200 μ M. The effect of cAMP on NAADP production and cGMP on cADPR synthesis should be compared at more physiological concentrations: Figure 8(C) shows that 30 μ M cAMP produces a 2-fold increase in NAADP production, just as 30 μ M cGMP enhances cADPR production 2-fold. In addition, 10 μ M forskolin was able to enhance NAADP production almost 2-fold (see Table 2), providing further evidence for a likely physiological effect of cAMP. NAADP synthesis was less sensitive to stimulation by cGMP than was cADPR production; cGMP had no significant effect above the control until

Figure 8 Concentration effect of cGMP and cAMP on cADPR and NAADP production

(A) cADPR production at 60 min, and (B) NAADP production at 120 min, were measured with increasing concentrations of cGMP or cAMP (x-axis, where X represents the respective nucleotide). The concentration of cADPR or NAADP, above the mean concentration produced in the absence of cXMP, is plotted on the y-axis. (C) Fold increase above control, where control production is 1-fold, for cADPR production in the presence of cGMP (solid line) and NAADP production in the presence of cAMP (broken line). All incubations were performed in the presence of 100 μM IBMX, and in addition, for NAADP production, 250 μM β-NADP⁺ and 7 mM nicotinic acid, and for cADPR production, 500 μM β-NADP⁺. Values are means ± S.E.M. for $n \geq 3$.

concentrations of 1 mM were applied. However, its ability to enhance NAADP production was apparent at these higher concentrations.

Figure 9 shows the effect of cAMP on NAADP production and cGMP on cADPR production for increasing substrate concentrations. These data have been transformed to give the double-reciprocal Lineweaver–Burk plot. The information obtained from the respective Lineweaver–Burk plots (i.e. the functions for the linear fits to the data) have been extrapolated to obtain approximate K_m and V_{max} values, as shown in Table 1. For NAADP production the apparent K_m values decreased by a factor of around 5 in the presence of cAMP, but there was apparently no significant change in the V_{max} with or without cAMP. In contrast, for cADPR production, the apparent K_m was not so markedly decreased in the presence of cGMP, differing by a factor of around 2, whereas V_{max} values for cADPR production with cGMP were increased to a significant degree.

In addition to the cAMP-mediated enhancement of NAADP production, 10 μM forskolin was shown to increase this activity, presumably via stimulation of adenylate cyclase in the egg homogenate (Table 2). Both the cAMP- and forskolin-mediated enhancement of NAADP production were abolished by Rp-

Figure 9 Increasing substrate concentrations for NAADP production in the presence and absence of cAMP, and cADPR in the presence and absence of cGMP

Egg homogenates (12.5%, v/v) were incubated with increasing concentrations of β-NADP⁺ (+ 7 mM nicotinic acid) for NAADP production, and β-NADP⁺ for cADPR. NAADP production was measured in the presence and absence of 500 μM cAMP (A), and a Lineweaver–Burk plot was derived from these data (B). cADPR production was measured in the presence and absence of 500 μM cGMP (C), and a Lineweaver–Burk plot was derived from this data (D). Values are means ± S.E.M. for $n = 3$ –6.

Table 1 Apparent K_m and V_{max} values obtained from linear fits to data transformed to Lineweaver–Burk plots

The values given are from the plots shown in Figures 9(B) and 9(D).

Incubation	$K_{m(app)}$ (mM)	V_{max} (pmol · h ⁻¹ · mg ⁻¹)	R^2 for linear fit to data
β-NADP ⁺ control	2.5	300	0.9992
β-NADP ⁺ + cAMP	0.5	300	0.9273
β-NADP ⁺ control	7.5	800	0.9996
β-NADP ⁺ + cGMP	4.1	1400	0.9990

cAMPs, which competitively blocks the action of cAMP at its target sites, including cAMP-dependent protein kinases.

DISCUSSION

In this study we demonstrate that the sea urchin egg possesses metabolic enzymes to synthesize and degrade NAADP. The fact that this cell is able to release calcium in response to NAADP, and that enzyme activities exist to control its intracellular levels, supports the idea that NAADP may function as an endogenous physiological Ca²⁺-mobilizing second messenger. The synthetic activity was able to produce concentrations of NAADP that are well within the range to release calcium in this system [10]. This is true even when production is considered under physiological, neutral pH conditions, when up to 100 nM NAADP was produced, which is close to the maximal concentration required for Ca²⁺ release [5,8]. Alongside the synthetic pathway there exists a degradative activity where up to 3 μM NAADP can be

Table 2 Effect of cAMP modulators on NAADP production

All incubations were performed in the presence of 100 μ M IBMX, 250 μ M β -NADP⁺ and 7 mM nicotinic acid, pH 7.0. The results are expressed as means $\pm$ S.E.M. and the significance was calculated for an increase over the control, using an unpaired Student's *t*-test, where *****P* < 0.0001, and ****P* < 0.001 (n.a. = not applicable; n.s.d. = not significantly different).

Condition	NAADP production (nmol $\cdot$ h ⁻¹ $\cdot$ mg ⁻¹)	<i>n</i>	Significance over control
Control	99.6 $\pm$ 3.4	6	n.a.
cAMP (500 μ M)	215.3 $\pm$ 17.8	6	***
cAMP (500 μ M) + Rp-cAMPS (500 μ M)	104.3 $\pm$ 14.9	6	n.s.d.
Forskolin (10 μ M)	162 $\pm$ 16.1	6	**
Forskolin (10 μ M) + Rp-cAMPS (500 μ M)	99.6 $\pm$ 24	3	n.s.d.

broken down. The production of NAADP is optimal at low pH levels, as found for NAADP synthesis by *Aplysia* ADP-ribosyl cyclase and CD38 [22].

The enzymic activities for NAADP production appear to be distinct from those that synthesize cADPR in terms of localization, temperature and pH optima. This suggests that different proteins may express these separate activities. cADPR production was predominantly cytosolic [4,25], whereas NAADP was produced mainly in the membrane fraction. This was unexpected, as it has been demonstrated that CD38 and *Aplysia* cyclase are able to use both β -NAD⁺ and β -NADP⁺ as alternative substrates to produce both cADPR and NAADP [22]. One possible model that could explain these findings is that the membrane-bound cADPR-producing activity (accounting for only about 10 % of the total activity under these experimental conditions) may be the site of NAADP synthesis. As there is a large cADPR hydrolase activity in this membrane fraction [25] (see also Figure 6B insert) it is possible that this NAADP-producing and cADPR-degrading activity may be similar to CD38.

NAADP exhibits unique inactivation properties, whereby low concentrations of this molecule desensitize any further response of the system to this same agent [10,11]. Therefore it is not possible to measure enzymic production of NAADP by bioassay in the same homogenate aliquot. Instead, synthesis was induced in separate samples of egg homogenates from those that were used to measure NAADP levels by the Ca²⁺-release bioassay. For the same reason, it is possible that in the intact cell, synthesis of NAADP may be compartmentalized at a site which is not directly associated with the Ca²⁺-release channels. Such compartmentalization may function to provide a rapid and highly localized release of this Ca²⁺-mobilizing agent.

It has previously been shown that cGMP enhances the production of cADPR in the sea urchin egg [20]. The results of this study provide new evidence that cAMP may be an important modulator for regulating NAADP production. cAMP was able to enhance NAADP production up to 9-fold in the whole egg homogenate, whereas cGMP increased cADPR production 2-fold under similar experimental conditions. The Lineweaver-Burk transformation of the data indicates that the predominant action of cAMP on NAADP production may be to enhance the apparent *K_m* for production, without affecting the *V_{max}*. In contrast, the action of cGMP on cADPR production is largely to increase the *V_{max}*, with a much smaller change in the apparent *K_m*. This may reflect important differences in the mechanism of action of cAMP and cGMP in enhancing NAADP- and cADPR-mediated Ca²⁺ release respectively.

The role of cGMP in activating cADPR production has been shown to be via a G-kinase [24], which may phosphorylate the cyclase enzyme. In the same way, cAMP may act on cAMP-dependent protein kinase and activate either the NAADP-

Figure 10 Scheme showing the possible mechanism of cADPR and NAADP-induced Ca²⁺ release: role of cGMP and cAMP in enzymic regulation

In the sea urchin egg, a soluble cytosolic protein (S) predominates to produce cADPR, and is induced by cGMP. A 'CD38-like' protein, which is able to produce NAADP, may occur in the membrane fraction (M), the activity being enhanced by cAMP. This protein may be analogous to CD38 due to its ability to synthesize NAADP and, in addition, this membrane fraction is capable of producing and degrading cADPR, although, as for CD38, the hydrolysis reaction predominates (the 'C' portion of the protein represents cyclase and 'H' represents hydrolase). Note that the single arrow indicating the action of cGMP and cAMP on the enzymes is shown for simplicity and is not assumed to be a single step. RyR is the ryanodine receptor and NAADP-R is the 'NAADP receptor', both of which gate Ca²⁺.

producing enzyme directly, or an additional regulatory element, since both forskolin and cAMP-mediated enhancement of NAADP synthesis were blocked by Rp-cAMPS, which inhibits cAMP target proteins including kinases.

Based on the data obtained in this study, and on our knowledge of cADPR and NAADP metabolism in the sea urchin egg and other systems [16], it is interesting to speculate that possible similar proteins that may be involved in the signal-transduction pathways mediating cADPR- and NAADP-induced calcium release. The membrane fraction of the sea urchin egg possesses the machinery to produce NAADP, to synthesize low levels of cADPR and to degrade cADPR efficiently. These enzyme activities are similar to those of CD38 [16,22], so it is possible that a bifunctional CD38-like protein, which can be stimulated by cAMP, may occur in the sea urchin egg membranes. The cADPR synthesizing activity, on the other hand, predominates in the cytosol of the egg and is differently regulated by cGMP [20] and may represent a novel cytosolic form, perhaps more closely related to the *Aplysia* ADP-ribosyl cyclase (Figure 10).

In summary, we have shown that the synthesis of two calcium-mobilizing pyridine nucleotides, NAADP and cADPR, are differentially regulated by cAMP or cGMP respectively. This information may provide an insight into the role of NAADP and cADPR in the transduction of signals generated by cell surface receptors via cAMP- and cGMP-dependent mechanisms.

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REFERENCES

- 1 Clapham, D. E. (1995) *Cell* **80**, 259–268
- 2 Berridge, M. J. (1993) *Nature (London)* **361**, 316–325
- 3 Clapper, D. L. and Lee, H. C. (1985) *J. Biol. Chem.* **260**, 13947–13954
- 4 Clapper, D. L., Walseth, T. F., Dargie, P. J. and Lee, H. C. (1987) *J. Biol. Chem.* **262**, 9561–9568
- 5 Lee, H. C. (1995) *J. Biol. Chem.* **270**, 2152–2157
- 6 Lee, H. C., Galione, A. and Walseth, T. F. (1994) *Vitam. Horm.* **48**, 199–257
- 7 Genazzani, A. A. and Galione, A. (1996) *Biochem. J.* **315**, 721–725
- 8 Genazzani, A. A. and Galione, A. (1997) *Trends Pharmacol. Sci.* **18**, 108–110
- 9 Genazzani, A. A., Mezna, M., Dickey, D. M., Michelangeli, F., Walseth, T. F. and Galione, A. (1997) *Br. J. Pharmacol.* **121**, 1489–1495
- 10 Genazzani, A. A., Empson, R. M. and Galione, A. (1996) *J. Biol. Chem.* **271**, 11599–11602
- 11 Aarhus, R., Dickey, D. M., Graeff, R. M., Gee, K. R., Walseth, T. F. and Lee, H. C. (1996) *J. Biol. Chem.* **271**, 8513–8516
- 12 Allen, G. J., Muir, S. R. and Sanders, D. (1995) *Science* **268**, 735–737
- 13 Masuda, W., Takenaka, S., Tsuyama, S., Tokunaga, M., Yamaji, R., Inui, H., Miyatake, K. and Nakano, Y. (1997) *FEBS Lett.* **405**, 105–106.
- 14 Rakovic, S., Galione, A., Ashamu, G. A., Potter, B. and Terrar, D. A. (1996) *Curr. Biol.* **6**, 989–996
- 15 Morita, K., Kitayama, S. and Dohi, T. (1997) *J. Biol. Chem.* **272**, 21002–21009
- 16 Lee, H. C. (1997) *Physiol. Rev.* **77**, 1133–1164
- 17 Summerhill, R. J., Jackson, D. G. and Galione, A. (1993) *FEBS Lett.* **335**, 231–233
- 18 Hellmich, M. R. and Strumwasser, F. (1991) *Cell Regul.* **2**, 193–202
- 19 Galione, A., McDougall, A., Busa, W. B., Willmott, N., Gillot, I. and Whitaker, M. (1993) *Science* **261**, 348–352
- 20 Galione, A., White, A. M., Willmott, N., Turner, M., Potter, B. and Watson, S. P. (1993) *Nature (London)* **365**, 456–459
- 21 Shubinsky, G. and Schlesinger, M. (1997) *Immunity* **7**, 315–324
- 22 Aarhus, R., Graeff, R. M., Dickey, D. M. and Walseth, T. F. (1995) *J. Biol. Chem.* **270**, 30327–30333
- 23 Samuels, M. L. (1989) *Statistics for the Life Sciences*, Prentice-Hall International (UK) Limited, London
- 24 Sethi, J. K. (1997) Ph.D. Thesis, Oxford University, U.K.
- 25 Wilson, H. L. and Galione, A. (1997) *The Pharmacologist* **39**, 410