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PHYSIOLOGICAL FUNCTIONS OF CYCLIC ADP-RIBOSE AND NAADP AS CALCIUM MESSENGERS

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■ **Abstract** Cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) are two Ca^{2+} messengers derived from NAD and NADP, respectively. Although NAADP is a linear molecule, structurally distinct from the cyclic cADPR, it is synthesized by similar enzymes, ADP-ribosyl cyclase and its homolog, CD38. The crystal structure of the cyclase has been solved and its active site identified. These two novel nucleotides have now been shown to be involved in a wide range of cellular functions including: cell cycle regulation in *Euglena*, a protist; gene expression in plants; and in animal systems, from fertilization to neurotransmitter release and long-term depression in brain. A battery of pharmacological reagents have been developed, providing valuable tools for elucidating the physiological functions of these two novel Ca^{2+} messengers. This article reviews these recent results and explores the implications of the existence of multiple Ca^{2+} messengers and Ca^{2+} stores in cells.

INTRODUCTION

The Ca^{2+}-mobilizing activities of cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) were first described in sea urchin eggs (1–4). These nucleotides have since been shown to be highly effective in releasing Ca^{2+} from internal stores in a wide variety of cells from protist and plant to human (reviewed in (5, 6)). The cyclic structure of cADPR based on X-ray crystallography is shown in Figure 1 (3). This novel cyclic nucleotide is derived from NAD (2). The site of cyclization is at the N1-position of the adenine ring, which is linked to the anomeric carbon of the terminal ribose (3). The nicotinamide group of the precursor NAD is released during the cyclization reaction. In contrast, NAADP is a linear metabolite of NADP (Figure 1). It is produced by exchanging the nicotinamide group of NADP with nicotinic acid (7). Despite the fact that the two molecules have clearly different structures and precursors, it is remarkable

that they are, nevertheless, synthesized by the same class of enzymes, which includes ADP-ribosyl cyclase and its homologue, CD38 (7). The mechanisms of the Ca^{2+}-releasing action of these two molecules are also completely independent and are different from that of inositol trisphosphate (IP_3) as well. Since the last comprehensive review of the mechanisms of Ca^{2+} signaling by these two Ca^{2+} messengers in 1997 (5), major advances have been made. The crystal structure of ADP-ribosyl cyclase has been determined, and its active site, as well as that of CD38, has been identified (8, 9). The cADPR-sensitive Ca^{2+} channels have been successfully reconstituted into lipid bilayers, and their characteristics indicate they are similar to the ryanodine receptor (10, 11). Elevation of cADPR levels following cellular activation has now been documented in protist, plant, and animal cells (12–14). Both cADPR and NAADP have been shown to mediate Ca^{2+} signaling in a wide variety of cells, and the functions they are involved in include fertilization, cell cycle regulation, insulin secretion, muscle contraction, nitric oxide signaling, neurotransmitter release, and activation of gene expression, as well as long-term depression. Cells thus possess multiple Ca^{2+} stores and multiple Ca^{2+} messengers for activating them. Ca^{2+} mobilization as a signaling mechanism is emerging as far more versatile and complex than the unitary view having IP_3 as the sole messenger. In the following sections, these recent advances will be reviewed with particular emphasis on the physiological functions of cADPR and NAADP.

STRUCTURES AND MECHANISMS OF ADP-RIBOSYL CYCLASE AND CD38

The presence of an enzymatic activity producing cADPR was first described in sea urchin egg homogenates (1, 2). This activity has since been shown to be ubiquitous [(15, 16) and reviewed in (17, 18)]. A soluble protein of about 30 kDa purified from *Aplysia* ovotestis was named ADP-ribosyl cyclase (19). Two homologs, CD38 and CD157, both of which are mammalian antigens, have been identified (20–22). Both share about 30% sequence identity with the cyclase and are capable of synthesizing cADPR from NAD. Additionally, CD38 can effectively hydrolyze cADPR to ADP-ribose (23–26). This class of enzymes is thus multifunctional, capable of catalyzing more than one reaction. Although CD38 was first described as a lymphocyte surface antigen, it has since been found in many other tissues, including eye and brain [(27, 28) and reviewed in (29–31)]. It is present not only on the surface of cells but also in intracellular organelles (32), including the nucleus (33–35). In liver nuclei, CD38 is localized by immuno-electronmicroscopy to the inner nuclear envelope (35).

In addition to these two CD molecules, a soluble form of the ADP-ribosyl cyclase has recently been purified from bovine brain (36). The brain enzyme is about 30 kDa, smaller than CD38, which is about 45 kDa. However, it is catalytically similar to CD38, possessing both cADPR-synthesizing and cADPR-hydrolyzing activities (36). Cyclase-like enzymes are present not only in animal cells but also in protist. A membrane-bound enzyme of about 41 kDa has been purified from

Euglena, which is catalytically similar to the *Aplysia* cyclase, possessing only cADPR synthesizing but not the hydrolyzing activity (37).

In addition to metabolizing cADPR, both the *Aplysia* cyclase and CD38 can also catalyze a transglycosylation reaction exchanging the terminal nicotinamide group of the substrate NADP with nicotinic acid and producing NAADP (7). Which catalytic path these enzymes take is determined by pH. In acidic pH and in the presence of nicotinic acid, NAADP is the predominant product, whereas at neutral or alkaline pH, the enzymes mainly cyclize NAD to produce cADPR (7). The unusual acidic pH dependency has led to the proposal that the NAADP-sensitive Ca^{2+} signaling may play a role in the endocytic pathway, which is composed of acidic organelles such as endosomes (5, 31). Thus, internalization of surface CD38 can expose the enzyme to acidic conditions conducive to NAADP synthesis.

How a single enzyme can use two different substrates and produce two structurally distinct Ca^{2+} messengers has been investigated using the *Aplysia* cyclase. The enzyme is a homodimer both in solution (38) and as crystals (39, 40). The dimer, formed by two bean-shaped monomers in a head-to-head fashion, resembles a donut with a central cavity. Figure 2 shows the secondary structures in one monomer and the van der Waals surfaces in the other. Four large β-structures constitute most of the carboxyl domain, which is separated by a central cleft from the amino domain containing α-helixes (39). The monomer is a very compact molecule packaged by five intramolecular disulfide bonds, three in the amino domain and two in the carboxyl domain.

Cocrystallization of the cyclase with nicotinamide, a substrate for the exchange reaction, shows that the active site is at a pocket near the central cleft (Figure 2) (8). In order to cyclize NAD, the substrate must be bound in a folded conformation such that the two ends of NAD are close enough for linkage. The pocket structure of the active site serves nicely to mold the substrate into such a configuration. Two tryptophan residues, Trp^{140} and Trp^{77}, are present lining the rim of the active site pocket and Trp^{140} is separated from the bound nicotinamide by only 2.8 Å (8). These two residues can serve to position the nicotinamide group and the adenine ring of NAD. Indeed, changing them to glycine using site-directed mutagenesis reduces the cADPR synthesizing activity by several thousand-fold (8).

The next catalytic step is likely to be the release of the nicotinamide group from NAD and the formation of an ADP-riboysl intermediate. The catalytic residue is shown by site-directed mutagenesis to be a glutamic acid, Glu^{179}. Changing it to even a conservative residue, such as aspartic acid or glutamine, renders the enzyme essentially inactive (8). As shown in Figure 2, the catalytic glutamate residue is situated deep inside the active site pocket. The ADP-ribosyl intermediate could be an oxocarbenium ion as has been proposed for similar enzymes (41–44). The anionic nature of the glutamate residue could serve to stabilize the cationic oxocarbenium intermediate. This function can also be shared by another nearby glutamate residue, Glu^{98}, which when mutated results in significant reduction in enzymatic activity (8). Alternatively, the intermediate could involve covalent ADP-ribosylation as has been shown in the case of CD38 (45), which is described below.

Intramolecular attack of the activated anomeric carbon of the ADP-ribosyl intermediate by the nitrogen at the 1-position of the adenine ring would result in cyclization, producing cADPR. If NADP is used as a substrate and nicotinic acid is present, nucleophilic attack of the intermediate by nicotinic acid would produce NAADP instead. At low pH, acidic residues around the active pocket are neutralized, allowing easier access of nicotinic acid to the active site and thus facilitating the exchange reaction. The fact that the cyclase can use either NAD or NADP as substrate suggests that the 2′-position of the adeninyl ribose of the bound substrate is pointing outward, away from the active pocket; thus, substrate binding would only be minimally affected, even if a bulky phosphate is present at the 2′-position, as in NADP.

Homology modeling of CD38 using the crystal coordinates of the cyclase indicates that the residues corresponding to the four critical residues of the cyclase described above are all clustered in a pocket that is similar to the active site of the cyclase (9). Glu^{226} of CD38, which corresponds to Glu^{179} of the cyclase, is also a catalytic residue because replacing it with even conservative residues inactivates the enzyme. Likewise, Trp^{125} and Trp^{189}, corresponding to the two positioning tryptophan residues in the cyclase, are also critical for the enzymatic activity (9). Therefore, CD38 and the cyclase are homologous not only in their primary sequences but also structurally. The catalytic scheme described above for the cyclase can account for the hydrolytic function of CD38 as well. It suffices to further assume that the active site of CD38 has high affinity for cADPR itself, such that cADPR can bind and be converted catalytically to the ADP-ribosyl intermediate. Accessibility of the active site pocket of CD38 to water may also be higher, allowing ready reaction of the ADP-ribosyl intermediate with water to produce ADP-ribose. Indeed, detailed kinetic analyses support the idea that a single intermediate is responsible for all the reactions catalyzed by CD38 (46). The nature of the intermediate has recently been investigated using a substrate-inhibitor of the enzyme, arabinosyl 2′-fluoro-2′-deoxynicotinamide mononucleotide (45). Incubation of CD38 with the compound results in the release of nicotinamide from it and the covalent attachment of the rest of the compound to the enzyme. The residue where the covalent modification occurs is identified as Glu^{226} (45), identical to the catalytic residue identified by site-directed mutagenesis (9). This result suggests that CD38 may be ADP-ribosylated covalently at Glu^{226} during normal catalysis when NAD is used as a substrate. The catalytic model described above thus presents a unified mechanism that accounts for all the known enzymatic properties of both the cyclase and CD38 (30).

PHARMACOLOGY OF CALCIUM SIGNALING MEDIATED BY cADPR AND NAADP

The class of pharmacological reagents that can selectively modify the cADPR-induced Ca^{2+} release is the modulators of the ryanodine receptor (RyR). Caffeine, a stimulator of the RyR, at high concentrations releases Ca^{2+} from the same stores

TABLE 1 Agonistic and antagonistic analogs of cADPR

Compound	Effect	Active concentration and other properties
cADPR	Agonistic	$EC_{50} \approx$ 18–48 nM (1, 83)
3-deaza-cADPR	Agonistic	$EC_{50} \approx$ 1 nM, metabolically stable (58)
2′A-deoxy-cADPR	Agonistic	$EC_{50} \approx$ 58 nM (176)
Cyclic aristeromycin diphosphate ribose	Agonistic	$EC_{50} \approx$ 80 nM, partial agonist, metabolically stable (177)
Caffeine	Agonistic	$EC_{50} \approx$ 5 mM, cell permeant (47)
8-NH_2-cADPR	Antagonistic	$IC_{50} \approx$ 10 nM, competitive (54)
7-deaza-8-Br-cADPR	Antagonistic	$IC_{50} \approx$ 0.7 μM, cell permeant and metabolically stable (55)
8-N_3-cADPR	Antagonistic	0.45 μM, photoaffinity labeling reagent (57)
8-Br-cADPR	Antagonistic	$IC_{50} \approx$ 1 μM (54)
3′A-*O*-methyl-cADPR	Antagonistic	$IC_{50} \approx$ 5 μM (176)
Ruthenium red	Antagonistic	50 μM (47)
Procaine	Antagonistic	1 mM (47)

as cADPR, and at low concentrations, it potentiates the effect of cADPR (47–49). Inhibitors of RyR, such as ruthenium red, procaine and Mg^{2+}, likewise block the action of cADPR (47, 48, 50). The effect of ryanodine itself is more complicated. In some systems, such as sea urchin eggs, ryanodine releases Ca^{2+} from the same stores as cADPR (47–49). In other systems, such as pancreatic acinar and neurons, ryanodine blocks the action of cADPR (51, 52). This dual effect of ryanodine is likely related to its known biphasic action on the RyR channel (53).

As shown in Table 1, the effective concentrations of the RyR modulators needed are relatively high. The discovery of ADP-ribosyl cyclase provides a versatile method to synthesize specific reagents and some of these are also listed in Table 1. In addition to NAD, the cyclase can cyclize a variety of NAD analogs to produce corresponding analogs of cADPR. The first series of useful cADPR analogs synthesized in this manner is the 8-derivatives, 8-NH_2-cADPR and 8-Br-cADPR; both are antagonists of cADPR (54). 8-NH_2-cADPR is much more effective than the 8-Br-derivative, but the latter is found to be cell permeant (55, 56). A more novel antagonist is 7-deaza-8-Br-cADPR, which is not only cell permeant but is also metabolically stable (55). Another antagonist that has useful properties is 8-N_3-cADPR, which is photoactive and has been used as an affinity probe for labeling the cADPR-receptor (57). Equally novel is the metabolically stable agonist, 3-deaza-cADPR, which is more than an order of magnitude more potent than cADPR itself (58).

The finding that the cyclase can also catalyze a base-exchange reaction to produce NAADP provides a similarly versatile method for synthesizing analogs of NAADP. Various analogs of NADP and nicotinic acid are used as substrates for the exchange reaction to produce a series of active NAADP analogs (59, 60)

TABLE 2 Active analogs of NAADP

Compound	Active concentration and other properties
NAADP	$IC_{50} \approx 0.5$ nM, $EC_{50} \approx 20$ nM (61, 62)
Etheno-NAADP	$IC_{50} \approx 60$ nM, $EC_{50} \approx 5\ \mu$M, fluorescent (60)
Etheno-aza-NAADP	$IC_{50} \approx 60$ nM, $EC_{50} \approx 2.5\ \mu$M, fluorescent (60)
3PSA-ADP	$IC_{50} \approx 0.5\ \mu$M, $EC_{50} \approx 3\ \mu$M (59)
Deamino-NAADP	$IC_{50} \approx 0.5\ \mu$M, $EC_{50} \approx 10\ \mu$M (59)

and some of these are listed in Table 2. A very unusual property of NAADP is that it can induce effective desensitization of the Ca^{2+} release mechanism even at subthreshold concentrations (61, 62). In this manner, NAADP can function as its own specific antagonist with an IC_{50} in the 1 nM range. All of the active analogs produced so far show a similar property as listed in Table 2 (59, 60). Two of these analogs, etheno-NAADP and etheno-aza-NAADP, are fluorescent (60) and potentially can be used to visualize the NAADP-receptor. In addition to the listed analogs, Bay K 8644 ($IC_{50} = 30\ \mu$M) and L-type Ca^{2+} channel blockers, such as diltazem ($IC_{50} = 7\ \mu$M), nifedipine ($IC_{50} = 11\ \mu$M), and verapamil ($IC_{50} = 21\ \mu$M), can also block the NAADP-induced Ca^{2+} release (63). The required concentrations of these Ca^{2+} antagonists are relatively high, however.

Inhibitors of the cyclase and CD38 are also available. Nicotinamide is cell permeant and inhibits the cyclase activity with an IC_{50} of about 1.5 mM (64, 65). It does not, however, truly inhibit but forces the reversal of the reaction. The cyclase is a reversible enzyme such that NAD is produced from cADPR in the presence of a high concentration of nicotinamide (23). Indeed, under the condition where the cyclase appears inhibited, there is a rapid exchange of the added nicotinamide with that of the NAD (7). Nevertheless, the membrane permeability of nicotinamide makes it a useful reagent. A much more potent inhibitor of CD38 is available. Arabinosyl 2′-fluoro-2′-deoxynicotinamide mononucleotide inhibits the enzyme with a K_i of 169 nM (45).

The battery of reagents described above thus provides valuable tools for pharmacologically dissecting the Ca^{2+} signaling pathways mediated by cADPR and NAADP, which are crucial in understanding the physiological functions of these novel Ca^{2+} messengers.

PHYSIOLOGICAL FUNCTIONS OF cADPR AND NAADP

Modulation of the Ryanodine Receptor

The first indication that cADPR may be an endogenous modulator of the RyR comes from pharmacological evidence as described above (47–49). However, the

action of cADPR is found to be complex and requires accessory proteins. Photoaffinity labeling shows that cADPR binds specifically to a 140-kDa protein (57), smaller in size than the RyR. Another cofactor is calmodulin, which greatly enhances the Ca^{2+}-releasing effect of cADPR (49, 66–69). Similar stimulation of muscle RyR by calmodulin has also been reported (70, 71).

More direct evidence that the target of cADPR is the RyR comes from reconstituting the cADPR-sensitive channels isolated from two species of sea urchin eggs into lipid bilayers. The reconstituted channels are Ca^{2+} selective and show concentration dependence on cADPR (10, 11). The conductance of the channel from one species is very similar to the mammalian RyR and its cADPR-dependency is found to require the presence of a dialyzable factor (11). The channel from the other species of sea urchin has somewhat smaller conductance, but the pharmacology is similar to the mammalian RyR nonetheless, being blocked by inhibitors of RyR and enhanced by caffeine. In both cases, the Ca^{2+}-conducting activity of the cADPR-channel is blocked by W7 and trifluoperazine, indicating it is dependent on calmodulin (10, 11), which is consistent with the results described above (49, 66–69).

Both cardiac and skeletal RyR channels have been reported to be activated by cADPR (72–74). Similarly, cADPR is found to increase the Ca^{2+} sensitivity of the third type of RyR isolated from diaphragm muscle and reconstituted into bilayers (75). Despite this body of positive evidence, the subject remains controversial because of negative results reported earlier (70, 76, 77). Part of the controversy is related to the fact that, as described above, the action of cADPR on the RyR requires accessory proteins. Conditions used to isolate and reconstitute the mammalian RyR that are not optimal for retaining these necessary factors can yield variable results.

Instead of relying on the difficult technique of reconstitution, a more informative approach is therefore to assess the effect of cADPR on RyR in more intact systems. It is thus convincing that in permeabilized clonal PC12 cells, the responsiveness to cADPR is strictly correlated with expression of the cardiac RyR, and cell lines devoid of RyR are not responsive (78). Even more direct evidence comes from analyzing the Ca^{2+} sparks in intact cardiac myocytes, which are due to elemental Ca^{2+} release from a single or a few RyR channels (79). The frequency of these Ca^{2+} sparks is found to be enhanced by cADPR (80, 81).

These results are further supported by measurements of ryanodine binding in detergent permeabilized parotid acini, which possess all three types of RyR (82). The distribution of the RyR can be visualized using BODIPY-ryanodine, a fluorescent analog, and both cADPR and ryanodine competitively inhibit the fluorescent staining in a similar manner (82). Analogous results are observed in microsomes isolated from T-lymphocytes. Ryanodine binds specifically to these membranes and the binding is modulated by cADPR in a concentration-dependent manner (14). Taken together, these results that come from the use of more native systems provide very convincing evidence that the target of cADPR is indeed the RyR.

Elevation of cADPR Levels Following Cell Activation

If cADPR is indeed a second messenger for Ca^{2+}, it is expected that its cellular levels would increase following activation by primary agonists. The first assay described for cADPR is a bioassay based on its Ca^{2+}-releasing activity in sea urchin egg homogenates (1, 83). Using this assay, the endogenous levels of cADPR in several rat tissues are determined, demonstrating cADPR is naturally occurring (84). The sensitivity of the bioassay is later improved using caffeine to enhance the Ca^{2+}-releasing activity of cADPR (48, 85). Another assay for cADPR is the radio-receptor assay based on the high affinity and specific binding of cADPR to egg microsomes (17, 86). Antibodies against cADPR, raised in rabbits (87) and chickens (88), are now available and a highly sensitive radio-immuno assay has been developed.

Human leukemic HL-60 cells are progenitors that can be induced to differentiate into different hemopoietic linages such as monocytes or granulocytes. Progressive accumulation of cellular cADPR as measured using the radioimmuno-assay is found to accompany monocytic differentiation induced by retinoic acid (87). The elevation of cADPR is specific for retinoic acid; other inducers of differentiation, such as cAMP, produce no such effect. Similar to retinoic acid, another cell-permeant agonist, nitric oxide, can likewise produce elevation of cADPR levels. This has been observed in both neuroscretory PC12 cells (78) and hippocampal slices (89). As will be discussed later, the effect of nitric oxide is mediated through activation of the ADP-ribosyl cyclase activity by the cGMP-dependent protein kinase. In pancreatic islets, stimulation of insulin secretion by glucose is also accompanied by cADPR elevation (90). In this case, it has been proposed that the action of glucose is due to glycolytic production of ATP, which in turn modulates the ADP-ribosyl cyclase activity (91).

The agonists described above all have their primary targets inside the cells. Agonists that are cell impermeant and act through stimulation of surface receptors can also elevate cellular cADPR levels. This is the case in T-lymphocytes. Stimulation of the T-cell receptor/CD3 complex by specific antibody OKT3 induces increases in cytoplasmic Ca^{2+} concentration and a sustained elevation of cADPR levels (14). In intestinal longitudinal muscles, activation of the surface cholecystokinin receptor likewise stimulates ADP-ribosyl cyclase activity, which is blocked by an antagonist of the receptor (92). Similar stimulation of the cyclase is seen in adrenal chromaffin cells following activation of the acetylcholine receptor (69). In all of these cases, Ca^{2+} influx accompanies surface receptor activation. It has been proposed that the influx may be responsible for stimulating the cyclase activity (69, 92). Alternative evidence suggests that the activation of the cyclase by surface receptors can be linked through a G-protein. Thus, treatment of membranes isolated from NG108 cells with a muscarinic agonist stimulates cADPR synthesis by 2- to 3-fold, which is inhibited by prior incubation with cholera toxin (93). A similar stimulatory effect by an adrenergic agonist is seen in membranes isolated from cardiac myocytes, which is mimicked by GTP-γ-S and blocked by the toxin (94).

Agonist-induced elevation of cellular cADPR content is seen not only in animal cells but in plants and *Euglena*, a protist, as well. Treatment of the subepidermal cells of *Aurea* hypocotyls with abscisic acid, a hormone that regulates plants response to environmental cues, rapidly elevates cellular cADPR, which in turn causally activates abscisic acid-specific genes (13). Likewise, in *Euglena*, treatment with vitamin B12, an obligatory growth factor, induces rapid elevation of cADPR, preceding the increases in DNA synthesis and cell number (12).

From the results described above, an intriguing generalization is that many of the primary agonists that signal through the cADPR-pathway are cell permeant. They include a gaseous messenger, nitric oxide, metabolic factors such as glucose and vitamin B12, and agonists that target intracellular receptors, such as retinoic acid. This is in contrast to the IP_3-signaling pathway, which is predominantly linked to surface receptor activation. Cells may thus employ two separate Ca^{2+} messengers, cADPR and IP_3, to distinguish, respectively, permeant and impermeant signals. This distinction is clearly not absolute. As described above, surface receptor stimulation can, in some cases, activate the cADPR-pathway as well.

Mediation of Ca^{2+} Signaling by Nitric Oxide

The finding that the ADP-ribosyl cyclase in sea urchin eggs is activated by cGMP (95), which is known to be raised by nitric oxide, suggests that the cADPR- and nitric oxide-signaling pathways are linked (96). The cGMP-dependent stimulation of the egg cyclase requires ATP (97) and is inhibited by protein kinase inhibitors (95), indicating that it is likely to be mediated by cGMP-dependent kinase phosphorylation. Exposing live sea urchin eggs to nitric oxide activates Ca^{2+} mobilization, which can be blocked by 8-amino-cADPR, a specific antagonist of the cADPR-receptor (54), nicotinamide, an inhibitor of the egg cyclase (65), and cGMP-dependent kinase inhibitors (98), providing direct evidence that the two pathways are linked.

That nitric oxide can activate Ca^{2+} mobilization via the cADPR-pathway has also been observed in clonal PC12-16A cells, an RyR-expressing neurosecretory cell line (78). This suggests that the connection between nitric oxide and cADPR may have relevance in neuronal function (99). Indeed neurons, such as dorsal root ganglion neurons, are known to be responsive to cADPR (100, 101). Metabotropic glutamate receptor activation in these neurons evokes transient depolarization, Ca^{2+}-activated inward currents and rises in intracellular Ca^{2+}, which can be mimicked by intracellular photorelease of caged cGMP or caged cADPR (101). 8-amino-cADPR and a cyclase inhibitor, nicotinamide, inhibit the effects of both caged analogs. These results are consistent with the functional presence of the cGMP/cADPR-signaling pathway in neurons.

The possibility that this pathway may be involved in higher integrative functions of the nervous system, such as long-term depression (LTD), has been examined using embryonic cerebellar cultures derived from transgenic mice. The gene for the neuronal nitric oxide synthase in these mice is ablated. Although the

cADPR-mechanism is present and operational in the cultured cerebellar cells, no effect of cADPR or cGMP on LTD has been observed (102).

The issue has recently been reexamined using hippocampal slices from normal rats, a more natural paradigm for investigating LTD (56, 89). Treatment of slices with a nitric oxide donor readily induces LTD, which can be blocked by ryanodine or cell-permeant antagonists of cADPR, 8-Br-cADPR (54), or 7-deaza-8-Br-cADPR (55), indicating that the effect of nitric oxide is mediated by cADPR (56). In addition to nitric oxide, cGMP levels in brain slices can be raised biochemically using an inhibitor of the cGMP-selective phosphodiesterase in conjunction with an inhibitor of cAMP-dependent protein kinase (89). This treatment increases the cADPR levels in the slices and induces LTD, which can be similarly blocked by ryanodine or cADPR antagonists. Inhibitors of cGMP-specific protein kinase likewise prevent LTD induction either by nitric oxide or by biochemical elevation of cGMP (56, 89). These results are consistent with nitric oxide raising cGMP levels in hippocampal slices, which in turn stimulates phosphorylation of ADP-ribosyl cyclase by a cGMP-dependent kinase. The resulting elevation of cADPR and mobilization of the cADPR-dependent Ca^{2+} stores then induce LTD. As is described in the section after the next, this nitric oxide/cADPR-signaling pathway is operational not only in animal cells, but also in plants.

Cell Cycle Signaling in Euglena

Euglena is an unicellular protist. So far, it is the most evolutionarily primitive cell that has been reported to possess the cADPR-pathway. Its ADP-ribosyl cyclase is a 40-kDa membrane-bound protein that is catalytically similar to the soluble *Aplysia* cyclase (37). Unlike the mammalian CD38, the *Euglena* cyclase does not have the cADPR hydrolyzing activity. *Euglena* synchronized by light-dark cycle divide simultaneously at the onset of darkness. Its cyclase activity increases markedly correlating with DNA synthesis and prior to cell division (12). Cell division can also be synchronized by addition of vitamin B12, an essential growth factor, to quiescent cells deprived of the factor. The cellular levels of cADPR increase concomitant to an increase in the cyclase activity and precede cell division (12), which suggests a causal relationship. Isolated microsomes show that the cADPR-sensitive Ca^{2+} stores are present and functional in *Euglena*. These microsomes are responsive to cADPR, and Ca^{2+} release from them is inhibited by ruthenium red (12, 103). Likewise, caffeine can activate Ca^{2+} release from the same stores (103), indicating that the pharmacology is similar to the mammalian cADPR-mechanism.

Evidence that cADPR may be involved in regulating the cell cycle in mammalian cells has also been reported (104). HeLa and 3T3 cells transfected with human CD38, a cADPR synthesizing enzyme (described above), show elevation of intracellular cADPR, partial depletion of thapsigargin-sensitive calcium stores, and increase in basal free cytoplasmic calcium concentration. The cell doubling time of these cells is reduced to as much as 35% of the control cells that do not express CD38 (104). The consistent evidence between *Euglena* and mammalian cells strongly suggests that cADPR is an important regulator of cell cycle.

Activation of Gene Expression in Plants

The presence of cADPR-sensitive Ca^{2+} channels in plants was first demonstrated in isolated vacuolar membranes, by using a Ca^{2+} release assay and also by a patch-clamping technique (105). The pharmacology of the plant channel has been shown to be very similar to the mammalian RyR (106, 107).

The cADPR-pathway is now known to be important in regulating gene expression in plants. Treatment of plant cells with the hormone abscisic acid (ABA) elevates cADPR levels and activates specific genes. The gene expression can be blocked by 8-amino-cADPR (13), a specific antagonist of cADPR-receptor (54), and mimicked by microinjection of either cADPR itself or the *Aplysia* cyclase (13). The latter presumably would produce cADPR from endogenous NAD. Although IP_3 can activate the same type of gene expression, its antagonist, heparin, can only block the IP_3-induced but not the ABA-induced gene expression, indicating that cADPR is selectively linked to the ABA-signaling pathway (13). Although pharmacological evidence using U73122, a phospholipase C inhibitor, suggests that IP_3 could also be involved (108), the inhibitor is now shown to be nonspecific and, in fact, is inhibitory to the cADPR-mechanism as well (109, 110).

That cADPR can indeed elevate intracellular Ca^{2+} by mobilizing the vacuolar Ca^{2+} store is demonstrated directly in guard cells (111). A consequence of the Ca^{2+} changes is the reduction in turgor pressure of the injected cell and closure of the stoma. ABA can induce similar Ca^{2+} changes and stomatal closure (108, 111). Both nicotinamide, an inhibitor of the ADP-riboysl cyclase, and 8-amino-cADPR specifically inhibit the ABA-induced stomatal closure (111). cADPR is thus an important messenger that mediates at least two effects of ABA: a short-term effect of closing the stoma to reduce water loss and a long-term effect of expressing specific genes to combat the environmental stress.

Another important function in plants, in which cADPR plays a role, is mediating the activation of genes involved in the defense against pathogens. Virus infection of plants elevates nitric oxide synthase activity and induces expression of specific defense genes. The pattern of gene expression activated by viral infection is mimicked by treatment with a nitric oxide donor (112). Introducing either cGMP or cADPR into the cells also induces expression of these genes, which is blocked by ruthenium red (112), an inhibitor of the cADPR-dependent Ca^{2+} channels. These results are consistent with nitric oxide raising cGMP levels, which in turn leads to production of cADPR and Ca^{2+} mobilization. The same nitric oxide/cADPR-pathway thus operates in plants and rat brain (described above), indicating that it is a general signaling mechanism.

Ca^{2+} Signaling in Oocytes

Marine invertebrate eggs, such as sea urchin eggs, have long been favorite models for investigating mechanisms of Ca^{2+} signaling. Fertilization of the eggs is accompanied by a Ca^{2+} wave that starts at the sperm-egg fusion site and propagates across the entire egg. This wave serves as a triggering ionic signal that initiates an

intrinsic program, cumulating in cell division and the eventual development of a mature organism.

Both the cADPR- and NAADP-mechanisms were first described to occur in sea urchin egg homogenates (1, 2, 4). That they are functional in live eggs is demonstrated most convincingly using caged analogs of cADPR and NAADP specifically synthesized for the purpose (113, 114). The functional role of cADPR is assessed using specific inhibitors and is found to mediate, in conjunction with IP_3, the Ca^{2+} wave at fertilization, which can be blocked only by inhibiting both the cADPR- and IP_3-mechanisms (55, 115, 116).

Similar to sea urchin eggs, both *Ascidian* and starfish oocytes possess all three Ca^{2+} signaling mechanisms. Fertilization of *Ascidian* eggs is accompanied by a rapid decrease in membrane Ca^{2+} current, exocytosis and the initiation of a long-term oscillation of cytoplasmic Ca^{2+} concentration (117, 118). Each of these functions is selectively served by one of the three Ca^{2+} signaling mechanisms (118). Thus, intracellular infusion of cADPR readily induces the membrane current decrease and exocytosis. NAADP, on the other hand, can activate the current changes but not the exocytosis (118). The cytoplasmic Ca^{2+} oscillation is most readily mimicked by infusion of IP_3, which however, is ineffective in mediating either exocytosis or the current change (118).

In starfish oocytes, the cADPR- and IP_3-mechanisms are present both in the cytoplasm and the nucleus (119, 120). Microinjection of antibodies against calmodulin substantially inhibits the rapid nuclear Ca^{2+} elevation induced by photolyzing caged cADPR co-injected into the nucleus (119), indicating that the mechanism is dependent on calmodulin and agrees with that seen in sea urchin eggs (49, 66). In the presence of specific inhibitors of the cADPR- and IP_3-mechanisms, the pattern of Ca^{2+} changes associated with oocyte-maturation activated by the hormone, 1-methyladenine, is selectively modulated, indicating that both Ca^{2+} signaling mechanisms are involved (119, 120). Additionally, after maturation, the NAADP-sensitive Ca^{2+}-release mechanism is found to be enhanced and appears to be associated more closely with the plasma membrane (121).

Mammalian eggs, including those from mouse, pig and cow, are also responsive to cADPR (122–124). Similar to that observed in *Ascidian* oocytes (118), cADPR is particularly effective, even at nanomolar concentrations, in triggering the cortical exocytosis (122), but is less so in inducing a cytoplasmic Ca^{2+} change (123), which suggests a preferential association of the cADPR-Ca^{2+} stores with the egg cortex.

Ca^{2+} Signaling in Pancreatic Acinar Cells

In addition to the three invertebrate eggs described above, pancreatic acinar cells and brain microsomes also possess both the cADPR- (52, 125, 126) and NAADP-sensitive mechanisms (127, 128), in addition to the IP_3-mechanism. Treatment of the acinar cells with cholecystokinin (CCK) activates repeated Ca^{2+} spiking in the apical secretory pole that contains a high density of zymogen granules. The Ca^{2+} spiking can be mimicked by infusion of cADPR, and its antagonist,

8-amino-cADPR, can specifically block the activating effect of physiological concentrations of CCK (52, 129). Because it has not been possible to demonstrate production of IP_3 at this hormone concentration range, cADPR may be the primary Ca^{2+} messenger under this condition (130). Isolated zymogen granules can respond to cADPR with Ca^{2+} release, which suggests that cADPR may in fact have a direct role in mediating granule exocytosis (131). The relative importance of the cADPR- and IP_3-mechanisms, however, can be metabolically regulated. Thus, high cellular glucose inhibits the cADPR-mechanism but enhances the IP_3-mechanism, making it dominant (132).

Of the three Ca^{2+} messengers, NAADP is the most effective in the acinar cells, being active in the nanomolar range as compared to the micromolar range for cADPR and IP_3 (128). Similar to that found in sea urchin eggs, the NAADP-mechanism in acinar cells can be desensitized after exposure to a high concentration of NAADP itself (61, 62). Prior desensitization of the NAADP-mechanism blocks the hormone-induced Ca^{2+} spiking, indicating that it plays a role in the hormonal signaling as well (128).

A scheme has been proposed to describe how the three Ca^{2+}-signaling mechanisms are coordinated in the acinar cells (128). Accordingly, CCK first activates a small and highly localized Ca^{2+} release from the NAADP-sensitive stores, which by itself is not sufficient to trigger Ca^{2+} spiking but requires the amplification by the cADPR-sensitive stores via Ca^{2+}-induced Ca^{2+} release (CICR). Thus, 8-amino-cADPR, by blocking the cADPR-receptor, inhibits not only cADPR-induced spiking but also the NAADP- and CKK-induced spiking (128, 129). That the cADPR-antagonist can indeed inhibit CICR has been directly demonstrated in sea urchin egg microsomes (48, 54). The Ca^{2+} signal amplified by the cADPR-mechanism further activates Ca^{2+} mobilization from the IP_3-sensitive Ca^{2+} stores, which ultimately are responsible for initiating Ca^{2+} spiking. Consistent with this notion is the observation that heparin, by blocking the CICR-activation of the IP_3-stores, inhibits spiking induced by CCK, NAADP, cADPR and IP_3 (52, 128). This scheme thus proposes NAADP as a trigger, the cADPR-stores as an intermediate amplifier and the IP_3-stores as the ultimate activator of hormone-induced Ca^{2+} spiking.

Ca^{2+} Signaling in Cardiac and Smooth Muscle Cells

Myocytes contain an abundance of cardiac RyR. As described above, cardiac RyR reconstituted into bilayers is responsive to cADPR (72, 73). Transfection and expression of cardiac RyR in cells also confer cADPR responsiveness (78). Therefore, it is puzzling that an early report suggests that cADPR does not regulate Ca^{2+} release in myocytes (133). A series of subsequent studies shows otherwise (80, 81, 134–136). The crucial factor appears to be temperature. Myocytes need to be maintained at a physiological temperature of 36°C (136), which was not done in the earlier study (133). Perhaps the most direct and visual demonstration of the effects of cADPR in myocytes is the enhancement of the frequency of Ca^{2+} sparks (80, 81), which are due to elemental Ca^{2+} release from a group of RyR (79).

Intracellular release of cADPR from its caged analog stimulates both the frequency of Ca^{2+} sparks and the amplitude of the Ca^{2+} transient associated with the action potential (80). Prior infusion of 8-amino-cADPR blocks the enhancing effects of cADPR on Ca^{2+} sparks. In detergent permeabilized myocytes addition of cADPR produces similar enhancement of the spark frequency that is readily reversible (81). In both cases, the effect of cADPR requires a period of time to develop maximally, which suggests some intermediate processes are involved, such as binding to accessory proteins as described above.

In addition to myocytes, three types of smooth muscles, including trachea (137), coronary artery (138) and intestinal longitudinal muscle (92), are also responsive to cADPR. In permeabilized tracheal smooth muscle, acetylcholine activates a long-lasting Ca^{2+} oscillation, which can be blocked by 8-amino-cADPR (137). A single Ca^{2+} transient is produced by cADPR alone, but it greatly increases the frequency and amplitude of the Ca^{2+} oscillation induced by subsequent exposure to acetylcholine. By sensitizing the Ca^{2+}-release mechanism, cADPR can thus effectively modulate the effects of agonists.

Ca^{2+} Signaling in Lymphatic and Blood Cells

Various types of lymphatic and blood cells, including macrophages (139), natural killer cells (140), lymphoma cells (141), T-lymphocytes (14, 142–144), and mononuclear cells (145), have been reported to be responsive to cADPR.

Activation of the TCR/CD3 receptor complex of T-lymphocytes with a specific antibody elicits a fast increase in intracellular Ca^{2+} believed to be mediated mainly by IP_3, which is followed by a sustained elevation owing to Ca^{2+} influx (14). The cellular level of cADPR increases and remains elevated, correlating temporally with the sustained Ca^{2+} influx. The influx is inhibited by 7-deaza-8-Br-cADPR, a permeant antagonist of cADPR (55), in a concentration-dependent manner, indicating that cADPR is indeed responsible (14). It is proposed that cADPR mediates Ca^{2+} influx by depleting the internal stores (144, 146), which in turn activates the capacitative Ca^{2+} entry mechanism (147). That cADPR can indeed mobilize the internal Ca^{2+} stores is directly shown in permeabilized lymphocytes (142, 143, 148). Consistent with RyR being the target of cADPR is the demonstration that RyR, particularly type 3, is present in lymphocytes and that cADPR can modulate ryanodine binding in microsomes (14). The biological consequence of activating the TCR/CD3 complex is cell proliferation, which is accompanied by expression of markers such as CD25 and HLA-DR. 7-deaza-8-Br-cADPR effectively inhibits both cell proliferation and the expression of markers in a concentration-dependent manner without much cytotoxic effect (14). These results provide strong evidence that cADPR is a second messenger important in regulating lymphocyte functions.

Similar results on proliferation are found in mononuclear cells derived from cord blood (145). Treatment of mononuclear cells with a high concentration of cADPR results in permeation of the messenger into the cells, sustained elevation of intracellular Ca^{2+}, and increases in the colony output as well as colony size.

These effects can be blocked by a cADPR-antagonist, 8-NH_2-cADPR. A non-hydrolyzable analog, 3-deaza-cADPR (58), is even more effective than cADPR itself. These results, taken together with the cell cycle shortening in HeLa and 3T3 cells described above (104), further strengthen the evidence of the importance of cADPR in regulating cell growth and proliferation.

Regulation of Insulin Secretion in Pancreatic β-Cells

Pancreatic β-cells respond to high concentrations of glucose by secreting insulin. Evidence suggests that cADPR plays an important role in this process. The cADPR level in the islets is raised by glucose (90, 149). Permeabilized islets respond to cADPR and secrete insulin (126). Cyclic ADP-ribose can release Ca^{2+} from microsomes isolated from the islets as well (90, 149). Transgenic mice over-expressing CD38 (a cADPR-metabolizing enzyme described above) in their β-cells show elevated plasma levels of insulin, and their islets also secrete more insulin in response to glucose (150). Conversely, mice with their CD38 gene knocked out show lower insulin levels in the serum after challenging with glucose, and their islets likewise secrete less insulin in response to glucose (149). The cellular levels of cADPR in the CD38-ablated islets also fail to increase following exposure to glucose.

More intriguing is the detection of the presence of autoantibodies against CD38 in 10%–14% of diabetic patients. These antibodies can bind to and specifically modulate the enzymatic activity of CD38, which suggests that the cADPR-metabolizing enzyme is important in the development of diabetes (151, 152).

Despite this impressive series of experiments, the subject has been somewhat controversial (153–155). Part of the reason is related to the type of cells used in the studies. Islets from diabetic mice (*ob/ob*) and cultured β-cells (RINm5F) do not respond to cADPR (90, 153–155). These cells also secrete much less insulin in response to glucose and have a depressed level of CD38 and RyR (90, 156). Another source of variability could be the requirement of accessory factors, such as FK506-binding protein (157) and the calmodulin-dependent protein kinase II (67), which have been shown to be important in conferring the cADPR-response in β-cells. Conditions that do not preserve the correct functioning of these factors could produce negative results.

Potentiation of CICR and Transmitter Release in Neurons

The mechanism of cADPR-induced Ca^{2+} release as delineated in sea urchin eggs occurs via sensitizing the Ca^{2+}-induced Ca^{2+}-release (CICR) mechanism ((47, 48) and reviewed in (158)). In the presence of calmodulin, increasing the concentration of cADPR reduces the concentration of Ca^{2+} (or Sr^{2+}) required to activate CICR, such that at high enough concentrations of cADPR, even basal levels of Ca^{2+} in the nanomolar range are sufficient (48, 49, 66). This potentiation mechanism has been shown to also operate in intact bullfrog sympathetic neuron (51) and NG108-15 cells (159, 160). Thus, application of cADPR through a patch-clamp pipette substantially augments the action potential- or depolarization-induced rises

in intracellular Ca^{2+}. The potentiation is inhibited by ryanodine, and Ca^{2+} imaging shows that cADPR enhances the spatial spread of the Ca^{2+} signal triggered by the influx at the edge of the cell into the center (160).

Another important neuronal function that involves cADPR is neurotransmitter release. As described above, cADPR is particularly effective in activating exocytosis in both sea urchin (83) and *Ascidian* eggs (118). This is also the case in adrenal chromaffin cells, which are neurosecretory. Permeabilized chromaffin cells respond to cADPR and release acetylcholine, which is blocked by pretreatment of the cells with imperatoxin, an inhibitory toxin of RyR (69).

Analogous results are found in neurons. Liposomal delivery of cADPR to frog nerve-muscle preparations evokes a rapid increase in the number of quanta released, mainly owing to an increase in the number of functional sites without affecting other quantal parameters (161). In the buccal ganglion of *Aplysia*, microinjection of cADPR into a cholinergic presynaptic neuron rapidly increases both the intracellular Ca^{2+} and the postsynaptic response evoked by a presynaptic spike (162). Preloading the neuron with 8-amino-cADPR or ryanodine blocks the effects. Analyses of the postsynaptic responses show that the number of acetylcholine quanta released is increased following cADPR injection. Application of NAD, the precursor of cADPR, likewise increases the quantal release. Single-cell reverse transcriptase polymerase chain reaction confirms the presence of the cyclase in the presynaptic neuron (162). These results show that cADPR is effective in enhancing neurotransmitter release in both vertebrate and invertebrate neurons.

Ca^{2+} Signaling in Salivary and Lacrimal Gland Cells

Various preparations of the salivary gland have been used to investigate the effect of cADPR. Microsomes prepared from the parotid gland respond to cADPR as assayed using a $^{45}Ca^{2+}$ efflux technique (163). Permeabilized cells from both the submandibular and the parotid glands respond to cADPR in a concentration-dependent manner, and these effects are blocked by many of the inhibitors of RyR, including imperatoxin, ruthenium red, benzocaine, and high concentrations of ryanodine. The cADPR-induced Ca^{2+} rlease is potentiated by Sr^{2+} and low concentrations of ryanodine. Calmodulin enhances the cADPR-effect, whereas W-7 depresses it (164, 165). The release is also blocked by 8-Br-cADPR (82), an antagonist of cADPR (54). None of these agents affect IP_3-induced Ca^{2+} release. The pharmacology is thus essentially identical to that seen in sea urchin eggs (reviewed in (5, 166)). Infusion of cADPR into intact gland cells likewise activates Ca^{2+} release as measured by the Ca^{2+}-activated Cl^- current (167). In these gland cells, however, cAMP seems to have an intriguing modulating effect on the cADPR-induced Ca^{2+} release that is not observed in sea urchin eggs.

The expression of RyR in these gland cells is detected by reverse transcriptase-polymerase chain reaction and can be visualized using a fluorescent analog of ryanodine (82, 164). The RyR is localized primarily in the basal pole whereas the

IP_3-receptor is predominantly in the apical pole (82). Specific binding of ryanodine to microsomes is competitively inhibited by cADPR. Both ryanodine and cADPR, likewise, competitively inhibit the fluorescent staining (82), providing visual and direct evidence that the target of cADPR is the RyR.

Permeabilized cells from lacrimal acini, another fluid-secreting gland, have also been shown to release Ca^{2+} in response to cADPR, which is blocked by ryanodine (168, 169). Stimulation of the cells with phenylephrine activates Ca^{2+} release without IP_3 production. The Ca^{2+} release is likewise inhibited totally by ryanodine, which suggests that cADPR is the mediator of the α-adrenergic receptor activation (168).

Intercellular Ca^{2+} Signaling

Cells in tissues are commonly connected by gap junctions, forming communicating conduits through which ions and small signaling molecules can permeate. Whether cADPR can diffuse through the gap junctions and mediate intercellular Ca^{2+} signaling has been investigated in lens cells (110, 170). In permeabilized lens cells, cADPR specifically releases Ca^{2+} from internal stores, and the release is blocked by 8-amino-cADPR but not by heparin (170). Microinjection of cADPR into intact cells in monolayer culture not only elevates Ca^{2+} in the injected cell but also initiates a Ca^{2+} wave that spreads to several layers of adjacent cells (110). Co-injection of cADPR with a Ca^{2+} chelator suppresses the Ca^{2+} change in the injected cell but not the spreading of the Ca^{2+} wave to the adjacent cells, indicating it is cADPR and not Ca^{2+} that is diffusing to the adjacent cells. These results show that cADPR, like IP_3, can permeate through gap junctions and serve as an intercellular Ca^{2+} messenger.

Intercellular signaling can occur extracellularly in a paracrine fashion as well. CD38 is present on the surface of many cells (reviewed in (29, 31)). It is homologous to the cyclase not only in sequence but also structurally (9). As shown in Figure 2, the cyclase is a dimer with a central cavity that is about the size of a molecule of cADPR. The cavity is lined with charged and hydrophilic residues (31). It has been proposed that CD38 could serve as a permeating channel for cADPR (5, 31). CD38 reconstituted into liposomes can indeed catalyze the transport of cADPR into the vesicles during its enzymatic synthesis from NAD (171). Similar transport of cADPR against its concentration gradient is seen in resealed human red cell ghosts that contain endogenous CD38. HeLa cells transfected with CD38 exhibit a transient elevation of intracellular Ca^{2+} following exposure to NAD; this suggests that cADPR is synthesized and transported across the cell membrane by surface CD38 to mobilize Ca^{2+} from internal stores (171). Several cell types, including HeLa cells, are found to possess a transport mechanism at the cell membrane that allows NAD efflux (172). Thus, NAD can be released by these cells and serve a paracrine signaling function. Prolonged incubation of NAD with HeLa cells transfected with CD38 triggers endocytosis of surface CD38, which is accompanied by a sustained elevation of intracellular Ca^{2+} (172).

Evidence suggests that cytosolic NAD is transported into the endocytic vesicles and is converted by the vesicular CD38 to cADPR, which in turn, is transported back to the cytosol to effect mobilization of internal Ca^{2+} stores (172). This novel NAD/CD38-mechanism has been invoked to account for the topological paradox of ecto-CD38 affecting intracellular Ca^{2+} signaling (173).

SEPARATE BUT INTERACTING CALCIUM STORES

It is now clear that cells possess at least three independent mechanisms for mobilizing internal Ca^{2+} stores. It is generally known that the IP_3-mechanism is present mainly in the endoplasmic reticulum (ER). Fractionation studies show that the cADPR-mechanism co-purifies with the IP_3-mechanism and glucose-6-phosphatase, a marker for the ER (1, 4, 86). Because the ER is believed to be continuous with the nuclear envelope, it is also likely to contain both the cADPR- and IP_3-dependent Ca^{2+}-release mechanisms, as depicted in Figure 3. This is shown to be the case in the isolated liver nuclei (33, 35, 174), which contain CD38 as well (33–35). This strategic localization of the whole cADPR-signaling machinery in the nuclear envelope can allow the pathway to directly regulate gene expression.

The colocalization of the cADPR- and IP_3-mechanisms is not obligatory. For example, in parotid acinar cells, the two mechanisms are segregated to two opposite poles of the cell (82). Organelles other than the ER may also contain the cADPR-mechanism. This is the case for pancreatic zymogen granules, an exocytotic organelle (131). Sea urchin egg cortical granules, another exocytotic organelle, and cortical ER likewise contain the cADPR-mechanism (Figure 3). This is suggested by the fact that cortical microsomes incorporated into lipid bilayers exhibit cADPR-dependent Ca^{2+} channels (11). The cortical localization of the mechanism can also account for the remarkable effectiveness of cADPR in inducing exocytosis and modulating the plasma membrane Ca^{2+} channels as observed in *Ascidian* eggs (Figure 3) (118). Thus, segregating the cADPR-mechanism to a specific region of the cell can allow a localized Ca^{2+} signal to regulate a selective function, such as membrane current or exocytosis, without necessarily activating the whole cell, providing a mean for fine tuning of signaling.

The identity of the organelle containing the NAADP-mechanism is currently unknown. Fractionation studies indicate that the NAADP-stores are separable from the IP_3- and cADPR-stores as well as from the mitochondria (4). The stores appear to possess a novel Ca^{2+}-ATPase that is insensitive to thapsigargin (175). In *Ascidian* eggs, the NAADP-stores are segregated in the cortex, because their mobilization readily modulates membrane current but has very little effect on the cytoplasmic Ca^{2+} concentration. In contrast, activation of the IP_3-stores triggers a cytoplasmic Ca^{2+} oscillation without eliciting membrane events (118). This is another example of segregated Ca^{2+} stores regulating distinct and selective functions. Another effect of the cortical stores is to insulate the Ca^{2+} changes in the region.

The abundance of Ca^{2+}-pumps in the cortical Ca^{2+} stores can effectively buffer the Ca^{2+} in the cortex and minimize the influx to and from the cytosol (Figure 3).

Although the three types of Ca^{2+} stores are separate, they can nevertheless functionally interact. Thus, a localized increase in NAADP by photolyzing its caged analog using a focused laser beam can elicit a Ca^{2+} wave that propagates across the entire sea urchin egg (6). On the other hand, a global increase by whole-field photolysis evokes Ca^{2+} oscillation, which has been proposed to be due to interaction between the NAADP- and cADPR-stores (5, 61, 114). That mobilization of the NAADP-stores can affect modulation of the IP_3-induced Ca^{2+} oscillation has been observed in *Ascidian* eggs (118). In pancreatic acinar cells, results described above suggest that the Ca^{2+} released from the NAADP-stores serves as a triggering signal that is sequentially amplified by the cADPR- and IP_3-stores through the CICR mechanism (Figure 3) (128).

The presence of separate but interacting Ca^{2+} stores that respond specifically to distinct messengers provides cells with a versatile mechanism for signaling. Specific and selective functions can be regulated by mobilizing locally segregated stores. Globally, cell activation can occur through interaction between stores, generating propagative Ca^{2+} waves. Ca^{2+} signaling can be extended even beyond cell boundaries through gap junctions or through a paracrine mechanism. The versatility of Ca^{2+} mobilization as a signaling mechanism makes it perfectly suited for responding to a wide range of environmental stimuli.

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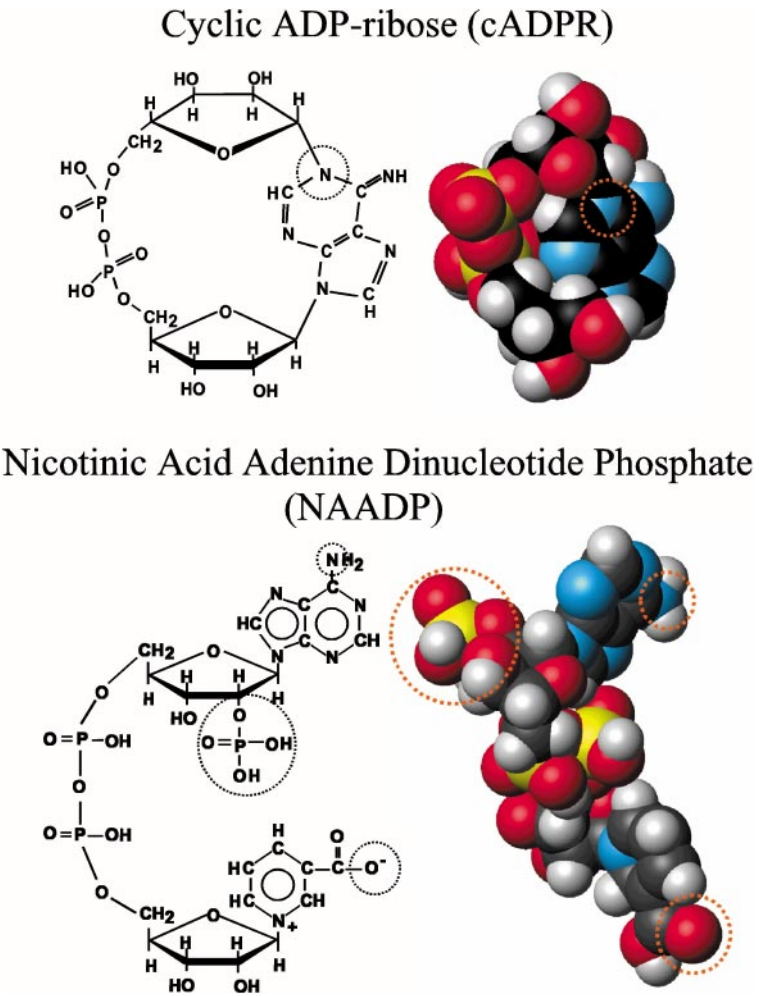

Figure 1 Structures of cADPR and NAADP. The N1-site of cyclization in cADPR is indicated by a dashed circle. The space-filling model is based on X-ray crystallography data (3). The three structural determinants of NAADP crucial to its Ca^{2+} releasing activity are also indicated by dashed circles (59). The space-filling structure of NAADP is based on the crystal coordinates of NADP bound to isocitrate dehydrogenase and modeled using ChemBuilder3D (30, 31). Color code: oxygen, red; hydrogen, white; carbon, black; nitrogen, cyan; phosphorous, yellow.

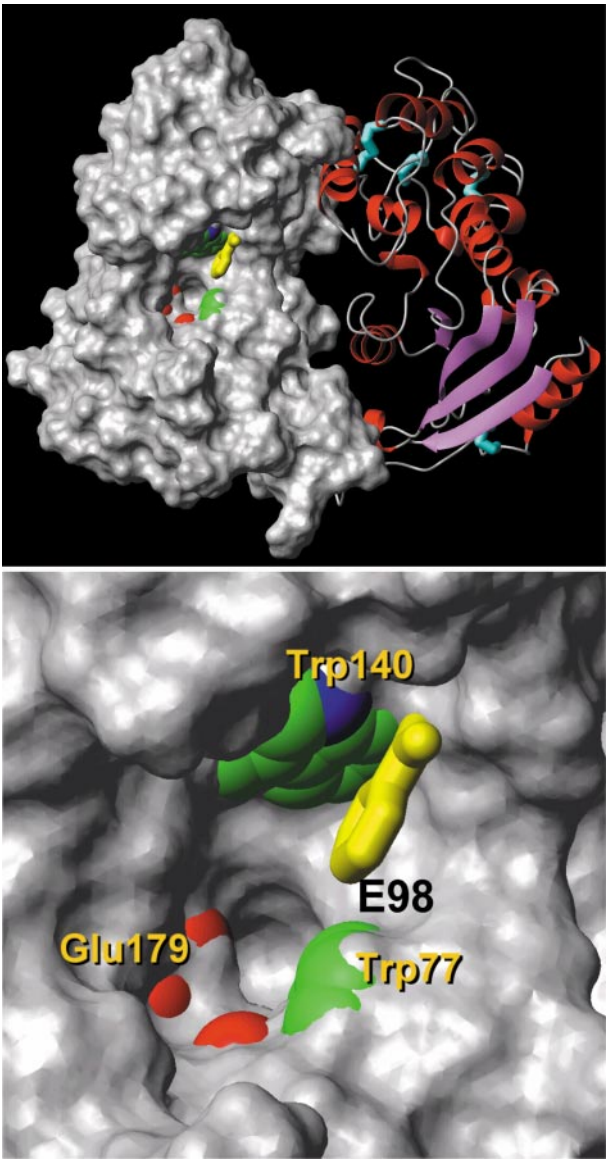

Figure 2 Crystal structure of ADP-ribosyl cyclase. The upper panel shows a cyclase homo-dimer with nicotinamide (yellow) bound to the active site. The structure is based on X-ray crystallography (8). The van der Waals surface is shown for the left monomer. The secondary structures are shown for the right monomer. Color code: α-helix, red; β-sheet, purple; coil, gray; disulfide bond, cyan. The lower panel shows the active site with three critical residues labeled. Color code: nicotinamide, yellow; carbon atom, green; oxygen, red; nitrogen, blue. All structures are rendered using the program MolMol (178).

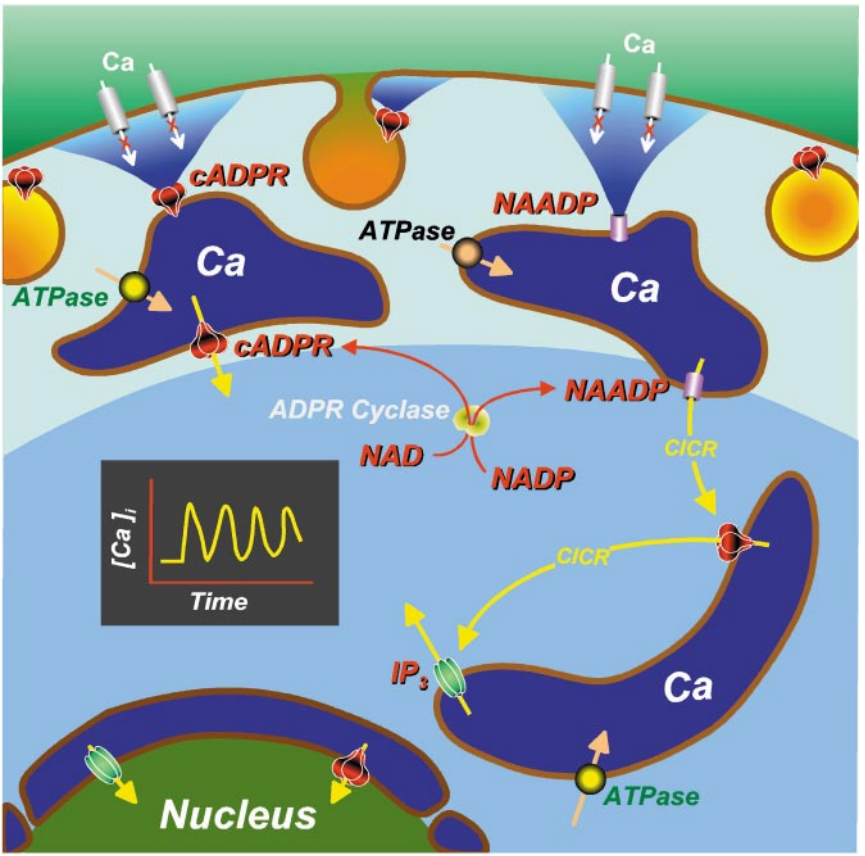

Figure 3 Separate but interacting Ca^{2+} stores. Mobilization of locally segregated Ca^{2+} stores regulates selective functions, such as membrane Ca^{2+} channels (white cylinders) and exocytosis. Interaction between Ca^{2+} stores via CICR can lead to propagative Ca^{2+} waves or oscillations, resulting in global activation of the cell. The abundance of Ca^{2+}-ATPase in the cortical stores effectively insulates the cortical region (light blue) from the Ca^{2+} changes in the cytosol (darker blue). A single enzyme, ADP-ribosyl cyclase, is responsible for synthesizing both cADPR and NAADP from NAD and NADP respectively. Representations: cADPR-mechanism, red tetramer; NAADP-mechanism, purple cylinder; IP_3-mechanism, green tetramer; thapsigargin sensitive Ca^{2+}-ATPase, yellow sphere; thapsigargin insensitive Ca^{2+}-ATPase, brown sphere.