NO-Mediated [Ca^{2+}]$_{cyt}$ Increases Depend on ADP-Ribosyl Cyclase Activity in Arabidopsis[1][OPEN]

S.M. Abdul-Awal, Carlos T. Hotta, Matthew P. Davey, Antony N. Dodd, Alison G. Smith, and Alex A.R. Webb*

Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, United Kingdom (S.M.A.-A., C.T.H., M.P.D., A.G.S., A.A.R.W.); Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna 9208, Bangladesh (S.M.A.-A.); Department of Biochemistry, University of Sao Paulo, Sao Paulo, CEP 05508/000, Brazil (C.T.H.); and School of Biological Sciences, University of Bristol, Bristol BS8 1TQ, United Kingdom (A.N.D.)

ORCID IDs: 0000-0002-8807-624X (S.M.A.-A.); 0000-0001-6511-5704 (A.G.S.); 0000-0003-0261-4375 (A.A.R.W.).

Cyclic ADP ribose (cADPR) is a Ca^{2+}-mobilizing intracellular second messenger synthesized from NAD by ADP-ribosyl cyclases (ADPR cyclases). In animals, cADPR targets the ryanodine receptor present in the sarcoplasmic/endoplasmic reticulum to promote Ca^{2+} release from intracellular stores to increase the concentration of cytosolic free Ca^{2+} in Arabidopsis (*Arabidopsis thaliana*), and cADPR has been proposed to play a central role in signal transduction pathways evoked by the drought and stress hormone, abscisic acid, and the circadian clock. Despite evidence for the action of cADPR in Arabidopsis, no predicted proteins with significant similarity to the known ADPR cyclases have been reported in any plant genome database, suggesting either that there is a unique route for cADPR synthesis or that a homolog of ADPR cyclase with low similarity might exist in plants. We sought to determine whether the low levels of ADPR cyclase activity reported in Arabidopsis are indicative of a bona fide activity that can be associated with the regulation of Ca^{2+} signaling. We adapted two different fluorescence-based assays to measure ADPR cyclase activity in Arabidopsis and found that this activity has the characteristics of a nucleotide cyclase that is activated by nitric oxide to increase cADPR and mobilize Ca^{2+}.

Cyclic ADP ribose (cADPR) is a signaling molecule that can evoke increases in the concentration of cytosolic free Ca^{2+} ([Ca^{2+}]$_{cyt}$) in plant and animal cells (Hetherington and Brownlee, 2004; Zhang and Li, 2006). In animals, cADPR is synthesized by a class of NADases called the ADP-ribosyl cyclases (ADPR cyclases). Metabolites of ADPR cyclase, including ADP-ribose (ADPR), cADPR, and nicotinic acid adenine dinucleotide phosphate, are all signaling molecules involved in Ca^{2+} signaling (Lee, 2001, 2006; Guse and Lee, 2008). In animals, both ADPR and cADPR stimulate Ca^{2+} influx through plasma membrane transient receptor potential channels (Perraud et al., 2001; Sano et al., 2001; Kraft et al., 2004). cADPR also mobilizes Ca^{2+} from the endoplasmic reticulum (ER) through an inositol 1,4,5-trisphosphate-independent mechanism (Galione et al., 1991; Lee and Aarhus, 1991, 1993; Galione, 1993, 1994; Lee, 1993), which most likely involves the modulation of ryanodine receptors (Li et al., 2001; Ozawa, 2001; Thomas et al., 2001). Nicotinic acid adenine dinucleotide phosphate mobilizes intracellular Ca^{2+} from lysosomal and/or acidic stores and is active in a variety of mammalian cell types (Lee, 2005).

In plants, neither ADPR cyclase nor an equivalent of the ryanodine receptor has been identified in genomic databases, even though ADPR cyclase activity and cADPR-evoked Ca^{2+} release from vacuoles and ER have been reported (Allen et al., 1995; Muir and Sanders, 1997; Leckie et al., 1998; Navazio et al., 2000; Sánchez et al., 2004). cADPR injected into guard cells causes stomatal closure (Leckie et al., 1998), and cADPR has been proposed to be involved in abscisic acid (ABA)-induced stomatal closure because 8-NH$_2$-cADPR, a competitor of cADPR signaling, and nicotinamide, an inhibitor of ADPR cyclase activity, both reduced ABA-induced stomatal closure (Leckie et al., 1998). The role of cADPR in ABA signaling also is supported by the statistically significant intersection between the sets of transcripts induced by ABA and cADPR (Sánchez et al., 2004). There is a similar intersection between transcript populations that are regulated by cADPR and the circadian clock, and together with circadian oscillations in the concentration of cADPR and an increased circadian period in the presence of nicotinamide, these data have led to the

proposal that cADPR forms a feedback loop in the Arabidopsis (*Arabidopsis thaliana*) circadian oscillator (Dodd et al., 2007).

The lack of orthologs for ADPR cyclase and RYR, and the limited characterization of their activities, have led to uncertainty concerning whether plants have a bona fide ADPR cyclase activity associated with Ca^{2+} signaling (Dodd et al., 2010). We sought to establish whether the reported ADPR cyclase-like activity in Arabidopsis has functional characteristics of an enzyme involved in the generation of cADPR to mobilize Ca^{2+} in plant signaling networks; specifically, we investigated if the enzyme activity was correlated with stimulus-induced increases in cADPR and also $[Ca^{2+}]_{cyt}$. We investigated the potential role of ADPR cyclase activity in nitric oxide (NO) signaling, because NO is a known regulator of the cADPR signaling pathway in animals (Galione et al., 1993; Willmott et al., 1996; Yu et al., 2000; Zhang and Li, 2006) and pharmacology suggests that NO-mediated increases in $[Ca^{2+}]_{cyt}$ are cADPR dependent in *Vicia faba* (Garcia-Mata et al., 2003). We reasoned that if cADPR is associated with NO signaling, as predicted by pharmacological studies, there might be NO-induced increases in Arabidopsis ADPR cyclase activity and NO-induced increases in the concentration of cADPR.

RESULTS

Pharmacological Identification of cADPR-Dependent Signaling Pathways in Arabidopsis

To investigate potential roles for cADPR in signaling in Arabidopsis, we investigated the effects of an antagonist of cADPR signaling on stimulus-induced increases of $[Ca^{2+}]_{cyt}$ in response to cold, NaCl, hydrogen peroxide (H_2O_2), and NO. We selected nicotinamide as a suitable antagonist because it is a metabolic by-product of cADPR production that acts as an inhibitor through product inhibition and enzyme reversal described by basic Michaelis-Menten kinetics. This simple pharmacology is easier to interpret than that based on analog compound chemistry, and we previously demonstrated dose-dependent inhibition of Arabidopsis ADPR cyclase activity by nicotinamide (Dodd et al., 2007). Nicotinamide also inhibits other NADases, including poly-ADP ribose polymerases and SIRTUINS, through the same product inhibition; however, neither of those enzymes has a known role in Ca^{2+} signaling, so an effect of nicotinamide on stimulus-induced $[Ca^{2+}]_{cyt}$ increases is indicative of ADPR cyclase activity (Galione, 1994). Cold treatment induced a transient increase of $[Ca^{2+}]_{cyt}$ in Arabidopsis that reached a peak of 440 ± 60 nM (mean $\pm$ SE; Fig. 1A), almost 3 times higher than the touch response evoked by room temperature water (152 ± 9 nM; Fig. 1A). In the presence of 50 mM nicotinamide, the cold-induced increase in $[Ca^{2+}]_{cyt}$ was slightly smaller, with the highest $[Ca^{2+}]_{cyt}$ peak of 358 ± 72 nM (Fig. 1A). A transient increase of $[Ca^{2+}]_{cyt}$ was detected in response to 10 mM H_2O_2 (peak $[Ca^{2+}]_{cyt}$ of 673 ± 45 nM; Fig. 1B). Preincubation with nicotinamide (50 mM) for 2 h reduced and slightly delayed the H_2O_2-induced $[Ca^{2+}]_{cyt}$ increase (peak $[Ca^{2+}]_{cyt}$ of 429 ± 20 nm; Fig. 1B). NaCl at 150 mM induced a large, rapid increase in $[Ca^{2+}]_{cyt}$ to a peak of 981 ± 229 nM (Fig. 1C), which was higher than cold water- and H_2O_2-mediated $[Ca^{2+}]_{cyt}$ responses. A partial reduction of the NaCl-induced $[Ca^{2+}]_{cyt}$ response was found when plants were incubated with nicotinamide

Figure 1. Nicotinamide abolished NO-induced $[Ca^{2+}]_{cyt}$ increases in Arabidopsis. A, Effect of cold water (4°C) on $[Ca^{2+}]_{cyt}$ in the absence or presence of nicotinamide (50 mM). B, Effect of H_2O_2 on $[Ca^{2+}]_{cyt}$ in the absence or presence of nicotinamide (50 mM). C, Effect of NaCl on $[Ca^{2+}]_{cyt}$ in the absence or presence of nicotinamide (50 mM). D, Effect of NO (SNAP) on $[Ca^{2+}]_{cyt}$ in the absence or presence of nicotinamide (50 mM). Twelve-day-old aequorin-expressing individual seedlings were incubated for 2 h with nicotinamide (50 mM). Cold water (4°C), 10 mM H_2O_2, 150 mM NaCl, and 300 µM SNAP (NO donor) were added at 15 s, and luminescence was measured for 896 s in a luminometer or multifunctional microplate reader. Data are presented as means of 12 biological replicates from three independent experiments ($n = 12$), and error bars represent SE. Arrows indicate the time of stimulation. RT, Room temperature.
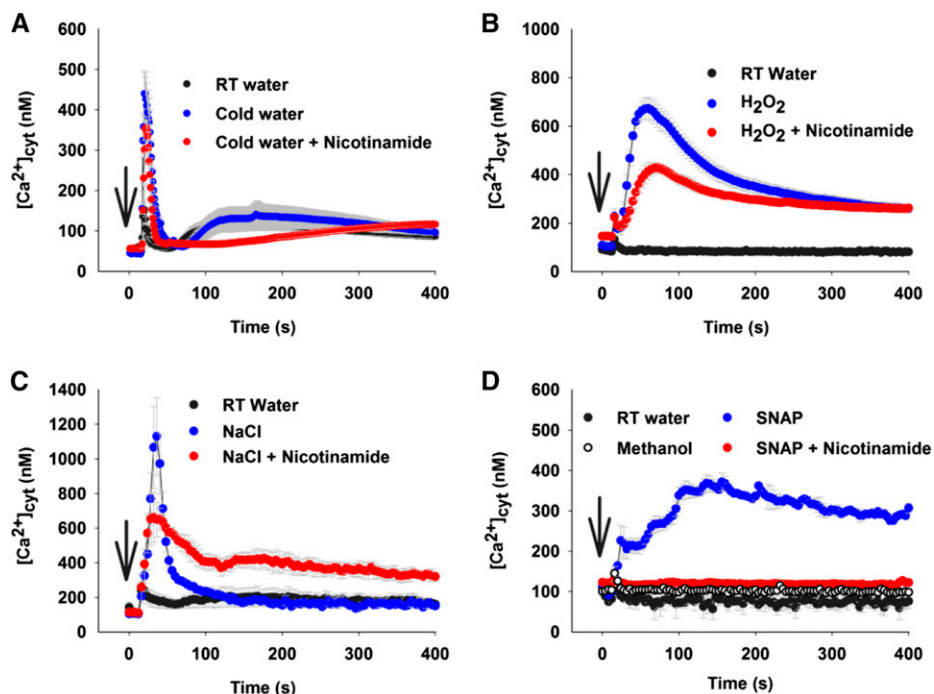
(50 mM; peak $[Ca^{2+}]_{cyt}$ of 662 ± 144 nM; Fig. 1C). *S*-Nitroso-*N*-acetylpenicillamine (SNAP) acts as an NO donor and triggers increases in $[Ca^{2+}]_{cyt}$ in Arabidopsis (Neill et al., 2002). SNAP at 300 μM elevated $[Ca^{2+}]_{cyt}$, and the increase was stable for 400 s, which was more prolonged than those induced by cold water, H_2O_2, and NaCl. The peak for SNAP-mediated $[Ca^{2+}]_{cyt}$ increase was 368 ± 18 nM (Fig. 1D), which was achieved 160 s after SNAP treatment, compared with the rapid responses to cold water, H_2O_2, and NaCl, in which the peak of $[Ca^{2+}]_{cyt}$ was induced within 15 to 30 s. Nicotinamide (50 mM) completely abolished SNAP-induced $[Ca^{2+}]_{cyt}$ increases (peak $[Ca^{2+}]_{cyt}$ of 123 ± 4 nM; Fig. 1D).

$[Ca^{2+}]_{cyt}$ Increases Induced by NO Are ADPR Cyclase Dependent

The inhibition of SNAP-induced increases in $[Ca^{2+}]_{cyt}$ by nicotinamide was suggestive of a role for ADPR cyclase in the elevation of $[Ca^{2+}]_{cyt}$ by NO. We performed a further set of experiments to confirm that the effects of SNAP were linked to NO production and not to an unintended side effect. First, we tested the effect of an alternative NO donor, sodium nitroprusside (SNP; Neill et al., 2002). At 5 μM, SNP induced sustained $[Ca^{2+}]_{cyt}$ increases that reached a plateau at 131 ± 6.6 nM (Fig. 2A). Increasing the concentration of SNP to 50 or 500 μM SNP had no further effect on $[Ca^{2+}]_{cyt}$, possibly because the experiment was performed in the dark, which limits the effectiveness of SNP (Rico-Lemus and Rodríguez-Garay, 2014). At 150 μM, SNAP induced sustained increases in $[Ca^{2+}]_{cyt}$ for 4 to 5 min until it reached a plateau at 195.1 ± 11.4 nM (Fig. 2B). Elevation of $[Ca^{2+}]_{cyt}$ by these two donors suggested that the effects were due to NO synthesis. This was confirmed by testing the effects of the NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Neill et al., 2002). Addition of 300 μM cPTIO 300 s after the addition of 150 μM SNAP decreased the $[Ca^{2+}]_{cyt}$ levels from the elevated value of 192.2 ± 13.6 nM to 115.3 ± 7.6 nM (Fig. 2C). Preincubation with 300 μM cPTIO reduced the $[Ca^{2+}]_{cyt}$ increase evoked by 150 μM SNAP to 145.3 ± 13.3 nM (Student's *t* test against 150 μM SNAP without 300 μM cPTIO, $P < 0.01$; Fig. 2D). Nicotinamide was equally effective in inhibiting NO-mediated increases in $[Ca^{2+}]_{cyt}$ if added before or after the NO donor SNAP (Fig. 2, E and F). Addition of 50 mM nicotinamide 300 s after the addition of SNAP reduced $[Ca^{2+}]_{cyt}$ levels from 215.8 ± 11.7 nM to 121.6 ± 5.6 nM; however, there was a long delay of over 60 s after the addition of nicotinamide before $[Ca^{2+}]_{cyt}$ decreased (Fig. 2E). This is supportive of the proposed role of nicotinamide in inhibiting the production of cADPR and possibly contributing to cADPR degradation by reversing the catalytic activity of ADPR cyclase to one of cADPR catalysis. SNAP addition after a prolonged incubation with nicotinamide resulted in a residual increase in $[Ca^{2+}]_{cyt}$ only to 139.1 ± 19.1 nM (Fig. 2F), demonstrating that the NO-induced increase in $[Ca^{2+}]_{cyt}$ might be almost completely dependent on cADPR. Osmotic effects of nicotinamide can be discounted, since an equimolar concentration

of mannitol was without effect (Fig. 2G). Preincubation for 300 s with $GdCl_3$ (the most effective blocker of Arabidopsis plasma membrane Ca^{2+} influx channels; Demidchik et al., 2002) at 1 mM, 10 times higher than required to inhibit NaCl-induced increases in $[Ca^{2+}]_{cyt}$ in the same assay (Tracy et al., 2008), did not reduce the $[Ca^{2+}]_{cyt}$ increase induced by 150 μM SNAP, which peaked at 198.7 ± 17.2 nM (Student's *t* test against 150 μM SNAP without 1 mM $GdCl_3$, $P = 0.81$; Fig. 2H), suggesting that plasma membrane influx of Ca^{2+} might not contribute to the response.

Nicotinamide Guanine Dinucleotide- and Nicotinamide Hypoxanthine Dinucleotide-Based Fluorescence Spectrometry Assays of Arabidopsis ADPR Cyclase Activity

The pharmacological manipulation of $[Ca^{2+}]_{cyt}$ is strongly indicative of a nicotinamide-sensitive component being required for NO-induced increases in $[Ca^{2+}]_{cyt}$ in Arabidopsis. To test if this increase is mediated by the activation of an ADPR cyclase-like activity, we assayed for ADPR cyclase activity based on the conversion of nonfluorescent nucleotide analogs of NAD into fluorescent cyclic nucleotides. Soluble total protein extracts of Arabidopsis have an enzymatic activity capable of converting the nonfluorescent NAD analog, nicotinamide guanine dinucleotide (NGD), to the fluorescent cyclic GDP-ribose (cGDPR; Fig. 3A). The synthesis of the cGDPR was dependent on the presence of NGD in both ecotype Columbia-0 (Col-0) and plants heterologously expressing ADPR cyclase from the sea slug *A. californica* (35S:*Ac* ADPR cyclase; Dodd et al., 2007). Furthermore, the rate of fluorescence increase was higher in protein extracted from the 35S:*Ac* ADPR cyclase plants (Fig. 3A).

The conversion of NGD to cGDPR was inhibited by NAD. In our assay, equal concentrations of NGD and NAD reduced the activity to 0.5-fold; however, an excess of NAD completely abolished the cyclization of NGD (Fig. 3B). While it is possible that NAD acts as a noncompetitive inhibitor, the reduction of the conversion of NGD to cGDPR by NAD is an expected characteristic of a nucleotide cyclase activity that favors NAD as a substrate to generate cADPR as a product. Animal ADPR cyclase is reversible under standard conditions, so we tested whether the inclusion of cADPR in the assay would inhibit the production of cGDPR from NGD. Addition of 25 μM cADPR reduced ADPR cyclase activity significantly ($P < 0.001$); however, higher concentrations of cADPR (up to 75 μM) did not cause any further changes in activity (Fig. 3C). The ADPR cyclase-like activity was protein dependent, being absent in boiled protein extracts (Fig. 3D). Based on these findings, we considered the fluorescence intensity increase to be representative of a bona fide ADPR cyclase activity. To determine the specific activity, we used commercial *A. californica* ADPR cyclase to generate a standard curve (Supplemental Fig. S1). This enabled us to estimate the specific activity in extracts of unstimulated Arabidopsis Col-0 to be around 0.01 to 0.015 units μg^{-1} total protein min^{-1} or units μg^{-1} protein min^{-1} (Fig. 3). An alternative

Figure 2. NO evokes short-term $[Ca^{2+}]_{cyt}$ increases. A, SNP was added at 60 and 360 s ($n = 5$ for each treatment) after the start of the experiment, and $[Ca^{2+}]_{cyt}$ levels were measured for 600 s. B, SNAP was added to provide a final concentration of 150 μM or 0.5% ethanol control was added 60 s after the start of the experiment, and $[Ca^{2+}]_{cyt}$ levels were measured for 600 s ($n = 19$). C, SNAP to a final concentration of 150 μM was added at 60 s, and 300 μM cPTIO was added at 360 s ($n = 21$). D, Seedlings were incubated with 300 μM cPTIO for 300 s before the start of the experiment, when 150 μM SNAP was added at 60 s ($n = 10$). E, SNAP at a final concentration of 150 μM was added 60 s after the start of the experiment, and 50 mM nicotinamide was added 300 s later ($n = 20$). F, Nicotinamide (50 mM) was added 60 s after the start of the experiment, and 150 μM SNAP was added 300 s later ($n = 8$). G, SNAP at a final concentration of 150 μM was added 60 s after the start of the experiment, and 50 mM mannitol was added 300 s later ($n = 5$). H, Seedlings were incubated for 300 s in 1 mM GdCl$_3$ before the start of the experiment. SNAP at a final concentration of 150 μM was added after 60 s (360 s; $n = 13$). Arrows indicate the time of each drug addition. Error bars represent SE.
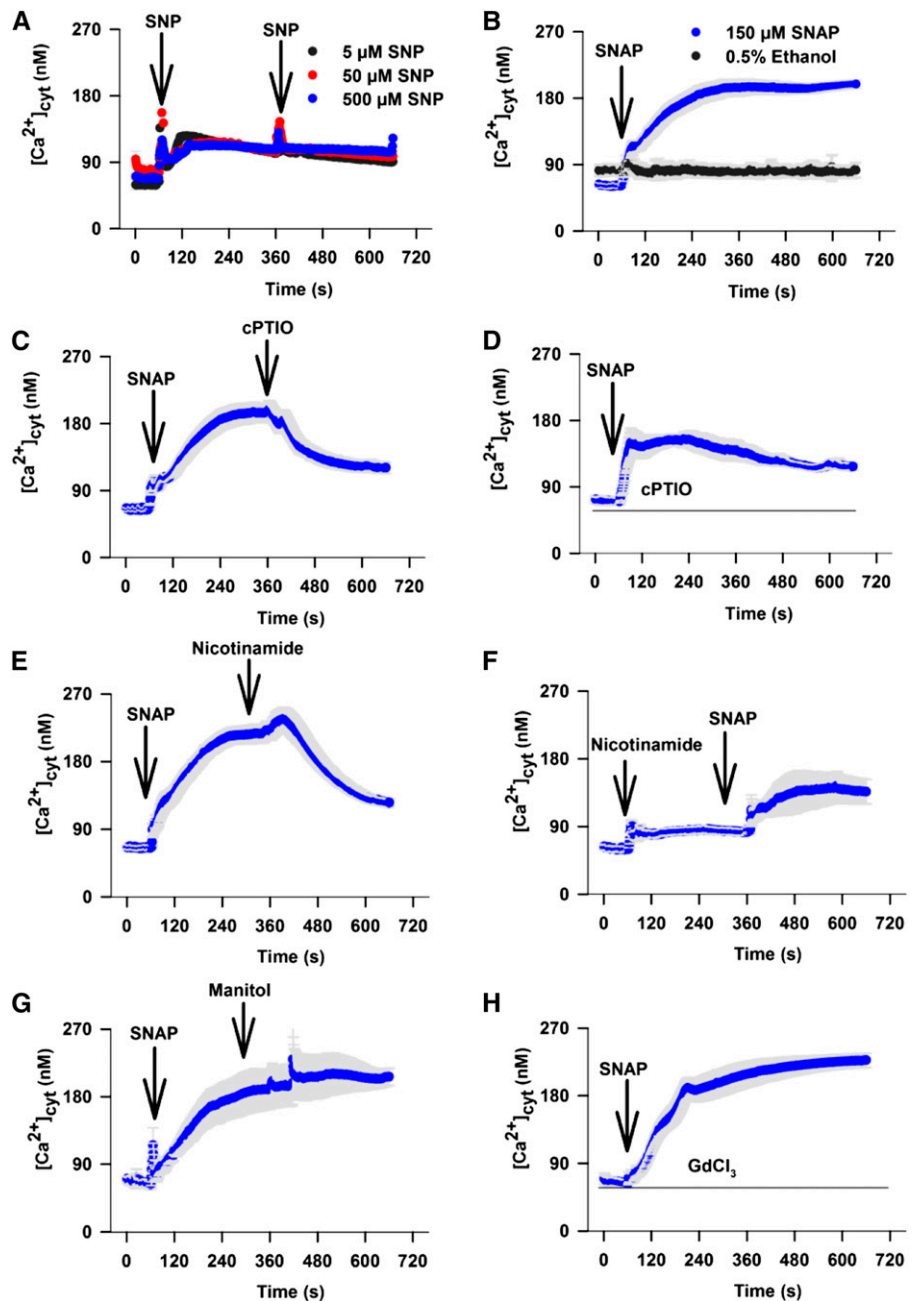
assay based on the conversion of nicotinamide hypoxanthine dinucleotide (NHD) to cyclic inosine diphosphoribose (Graeff et al., 1996) resulted in a very similar estimate of Col-0 ADPR cyclase activity (Fig. 3E), while, as expected, 35S:*Ac* ADPR cyclase plants had significantly higher ADPR cyclase activity of 0.027 ± 0.0008 units μg^{-1} protein min^{-1} ($P \leq 0.001$; Fig. 3E).

NO Is a Regulator of Arabidopsis ADPR Cyclase Activity

NO treatment of whole plants significantly increased the extractable ADPR cyclase activity using either NGD or NHD as substrate ($P \leq 0.001$), and the NO scavenger

cPTIO significantly reduced the effect of SNAP on extractable ADPR cyclase activity (Fig. 4, A and B). Similarly, adding SNAP to the extracted proteins also increased Arabidopsis ADPR cyclase activity, which was likewise reversed by cPTIO (Fig. 4B; $P \leq 0.001$). This demonstrates that NO can regulate ADPR cyclase activity in a cell-free manner.

To investigate whether physiologically relevant levels of NO can regulate ADPR cyclase activity in Arabidopsis, we measured it in lines carrying the *calmodulin-like24-4* allele, which results in constitutively high NO (Tsai et al., 2007). Both *cml24-4* and *cml23-3 cml24-4* plants had significantly higher extractable
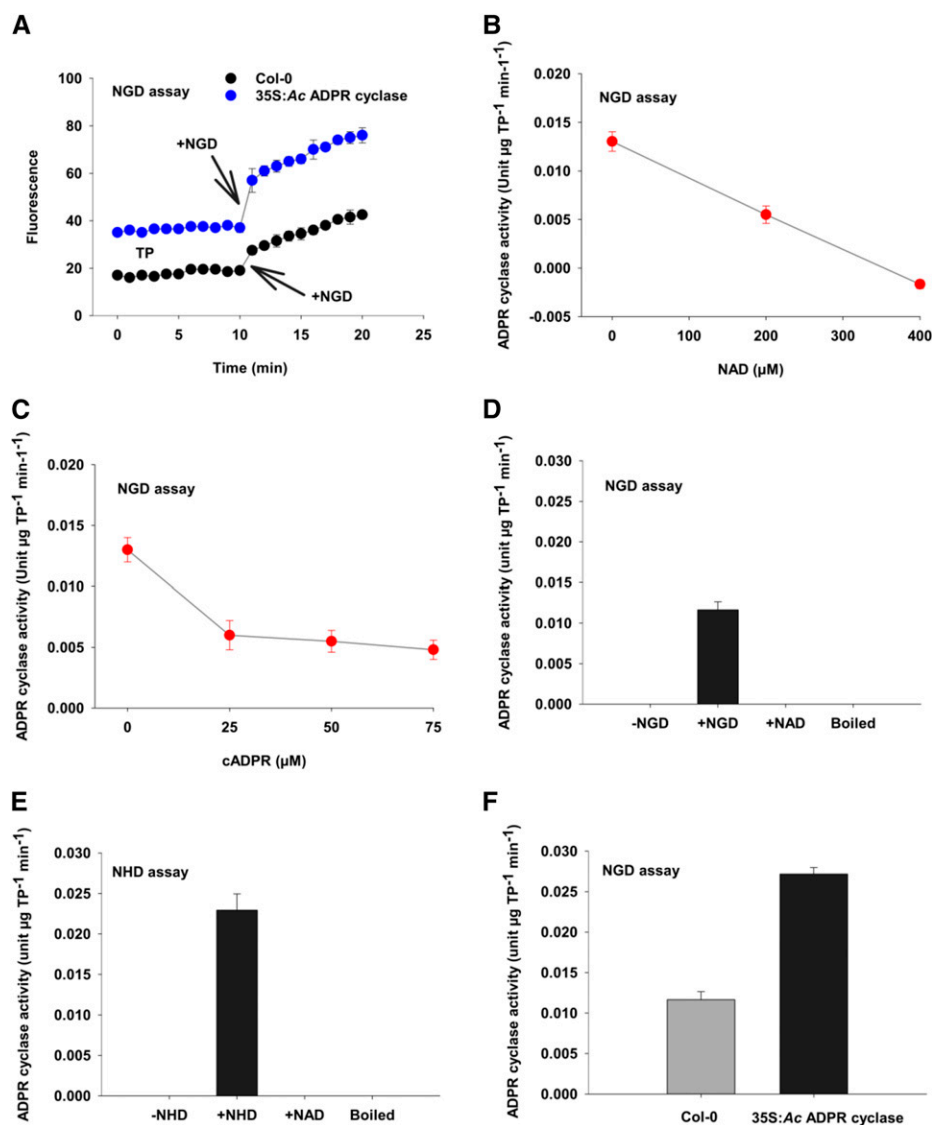
Figure 3. Identification of ADPR cyclase activity in Arabidopsis. A, Time course of ADPR cyclase activity in soluble protein extracts of leaves of Col-0 or plants transformed with 35S:*Aplysia californica* ADPR cyclase (35S:*Ac* ADPR cyclase). All components except substrate were added to the cuvette, then 200 μM NGD was added at 10 min, and fluorescence intensity was measured for another 10 min. B, ADPR cyclase activity of extracts of Col-0 leaves in the presence of NAD. NAD reduced the activity in a concentration-dependent manner. C, ADPR cyclase activity of Col-0 leaf extracts measured using NGD as a substrate (200 μM) in the presence of cADPR. D, Estimated ADPR cyclase activity in extracts of Col-0 based on the cyclization of NGD in the absence or presence of 200 μM NGD, 200 μM NAD as an alternative substrate, and when enzymatic activity had been inhibited by boiling for 10 min. E, Estimated ADPR cyclase activity in extracts of Col-0 based on the cyclization of NHD in the absence or presence of 200 μM NHD, 200 μM NAD as an alternative substrate, and when enzymatic activity had been inhibited by boiling for 10 min. F, Activity of ADPR cyclase in extracts of Col-0 and 35S:*Ac* ADPR cyclase plants calculated from a standard curve derived from *A. californica* ADPR cyclase (Supplemental Fig. S1). Fluorescence is in arbitrary units. Data are presented as means of three biological replicates of three independent experiments, and error bars represent SE. TP, Total protein.

ADPR cyclase activity compared with wild-type Col-0 plants ($P \le 0.001$; Fig. 4C).

ABA increases NO in guard cells (Neill et al., 2002); therefore, we tested the effect of this phytohormone on ADPR cyclase activity. Soluble protein extracts of Col-0 plants treated with 50 μM ABA had significantly higher ADPR cyclase activity of 0.026 ± 0.001 units μg^{-1} total protein min^{-1} ($P \le 0.001$; Fig. 4D) compared with untreated protein extracts of Col-0 plants (Fig. 4D). This activation appears to be physiologically relevant, because the activation by ABA was less than that due to the exogenous NO donor SNAP, which might be expected to cause very high levels of NO ($P \le 0.001$; Fig. 4D). NO-induced ADPR cyclase activity was inhibited by nicotinamide in a dose-dependent manner, with complete inhibition being achieved at 50 mM nicotinamide (Supplemental Fig. S2), consistent with the effect of nicotinamide on NO-induced increases in [Ca^{2+}]$_{cyt}$ (Figs. 1D and 2, E and F).

The activation of ADPR cyclase activity by NO was confirmed by the measurement of [cADPR] in Arabidopsis treated with 300 μM SNAP or 0.5% (v/v) methanol using a fluorescence-based coupled assay (Dodd et al., 2007). Before treatment, [cADPR] was 0.72 ± 0.09 pmol μg^{-1} protein (Fig. 5), and [cADPR] levels in the plants treated with the 0.5% (v/v) methanol control remained almost constant at all time points, varying from 0.47 ± 0.00 pmol μg^{-1} protein 30 min after the treatment to 0.89 ± 0.09 pmol μg^{-1} protein 60 min after the treatment (Fig. 5). The addition of 300 μM SNAP caused a fast increase of [cADPR] in the first 5 min, to 1.62 ± 0.34 pmol μg^{-1} protein, before slowly returning to resting levels at 60 min (0.77 pmol μg^{-1} protein; Fig. 5).

DISCUSSION

NO Increases [Ca^{2+}]$_{cyt}$ through a Pathway That Includes the Activation of ADPR Cyclase

We found that NO-mediated [Ca^{2+}]$_{cyt}$ increases were abolished by incubation with the NADase inhibitor

Figure 4. ADPR cyclase activity in response to NO. A, ADPR cyclase activity in the soluble protein extract of SNAP (300 μM)-treated Col-0 plants. B, Effect of 300 μM SNAP on ADPR cyclase activity of the protein extracted from untreated Col-0 plants. C, ADPR cyclase activity in soluble protein extracts of *cml24-4* and *cml23-2 cml24-4* plants. D, ADPR cyclase activity in ABA (50 μM)-treated protein extracts of untreated Col-0 plants. Soluble protein extracts were prepared from 4- to 5-week-old plants, and equal amounts of protein (145 μg) were used to measure the ADPR cyclase activity by NGD or NHD assay. Data are presented as means of three biological replicates of three independent experiments, and error bars represent SE. TP, Total protein.
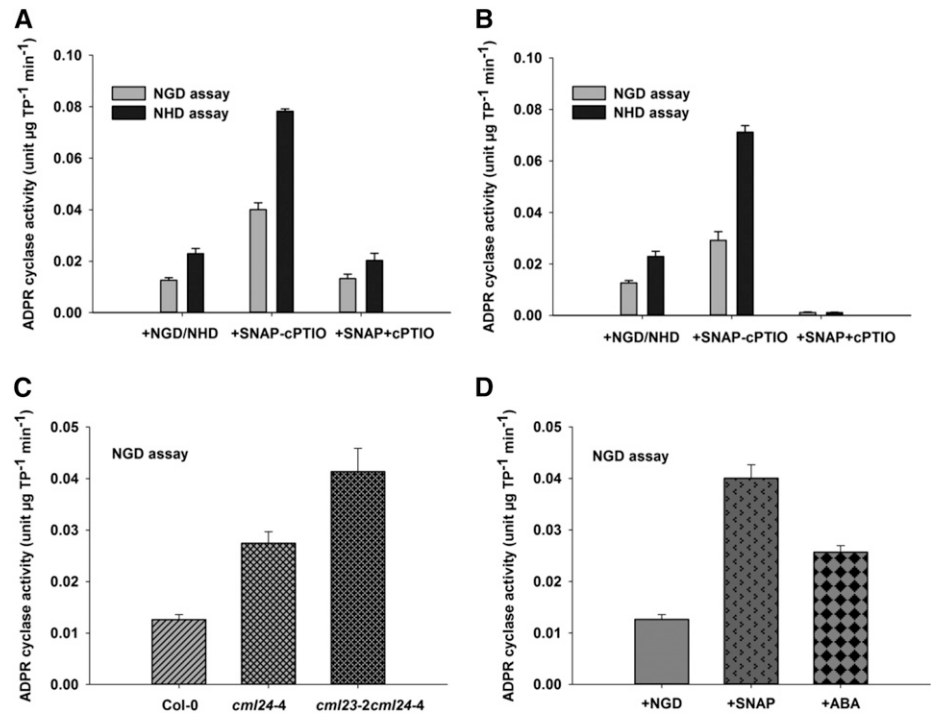
nicotinamide, that NO increases ADPR cyclase activity, and that NO stimulates the production cADPR, a Ca^{2+} agonist. These data and the insensitivity of NO-mediated increases in $[Ca^{2+}]_{cyt}$ to $GdCl_3$, an inhibitor of the plasma membrane-mediated influx of Ca^{2+}, lead us to conclude that the primary pathway by which NO increases $[Ca^{2+}]_{cyt}$ in Arabidopsis is through cADPR-mediated Ca^{2+} release from the ER and/or the vacuole, dependent on the activity of ADPR cyclase.

The conservation of the regulation of ADPR cyclase activity by NO between plants and animals could suggest a common ancestry for the pathway; alternatively, this might be an example of the convergent evolution of signaling in the plant and animal lineages. However, the lack of obvious orthologs for ADPR cyclase and ryanodine receptors in the Arabidopsis and other plant genomes makes it challenging to confirm either of these hypotheses. Our adaptation of ADPR cyclase activity assays for Arabidopsis, and the identification of both NO and *cml24-4* mutants as activators of ADPR cyclase, provide a tool set that might aid in the isolation of the ADPR cyclase protein and the identification of the corresponding gene. This might provide information concerning potential evolutionary features of cADPR-dependent NO-induced increases in $[Ca^{2+}]_{cyt}$. Our discovery that *cml24-4* plants have higher ADPR cyclase activity provides a potential genetic background to use in attempts to purify the enzyme. We have found that pharmacological tools can be used to activate and inhibit ADPR cyclase activity in a cell-free manner, which could be useful in confirming that a purified product represents a potential ADPR cyclase.

We found little evidence that cADPR signaling contributes to cold-, touch-, and H_2O_2-induced increases in

$[Ca^{2+}]_{cyt}$. Cold-induced increases in $[Ca^{2+}]_{cyt}$ are due to influx across the plasma membrane and efflux of Ca^{2+} from the vacuole (Knight et al., 1996), apparently through a cADPR-independent route. The analysis of H_2O_2-mediated $[Ca^{2+}]_{cyt}$ signals revealed that the initial increase of $[Ca^{2+}]_{cyt}$ was partially suppressed by 50 mM nicotinamide (Fig. 1B). However, this effect was much
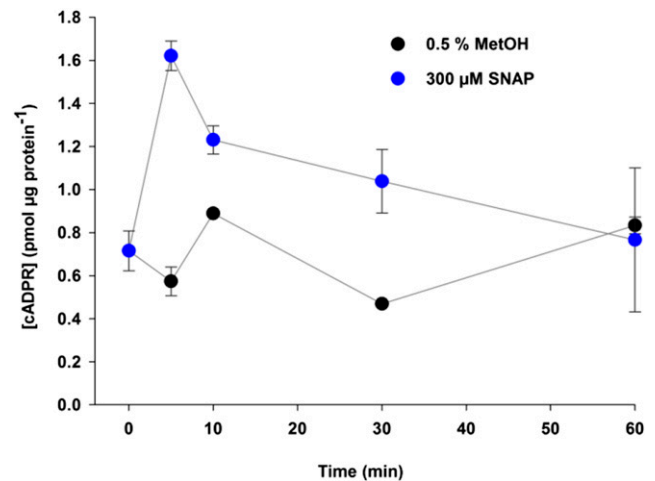

Figure 5. SNAP triggers [cADPR] accumulation in Arabidopsis. Three-week-old Arabidopsis seedlings grown in 12 h of light/12 h of dark were treated with 300 μM SNAP or methanol control (0.5% [v/v] MetOH) by flooding the plates for 1 min. Each plate contained an average of 20 seedlings, and all of them were harvested in each time point. Three independent replicates were harvested at the beginning of the time course and 5, 10, 30, and 60 min after drug treatment. [cADPR] was estimated by a coupled assay, and each sample was measured at least twice. Error bars represent SE.

less than observed for NO, and we conclude that the bulk increase in $[Ca^{2+}]_{cyt}$ in response to H_2O_2 is not ADPR cyclase dependent. $[Ca^{2+}]_{cyt}$ elevations in response to H_2O_2 treatment arise primarily through the activation of hyperpolarization-activated Ca^{2+}-permeable channels in the plasma membrane (Pei et al., 2000; Rentel and Knight, 2004). NaCl elevates $[Ca^{2+}]_{cyt}$ within very short periods in plants (Knight et al., 1997; Kiegle et al., 2000; Knight, 2000; Moore et al., 2002). We also detected immediate rapid responses of $[Ca^{2+}]_{cyt}$ to NaCl. Nicotinamide had some inhibitory effects but did not abolish NaCl-mediated $[Ca^{2+}]_{cyt}$ increases (Fig. 1C). Based on this finding and studies with inhibitors of plasma membrane Ca^{2+} influx (Tracy et al., 2008), it appears that NaCl-induced increases involve both influx across the plasma membrane and cADPR-mediated Ca^{2+} release from the ER or vacuole. Release of Ca^{2+} from multiple stores through different pathways might permit the oscillatory $[Ca^{2+}]_{cyt}$ signals induced by NaCl (Martí et al., 2013), which is a result of spatial heterogeneity (Tracy et al., 2008) and cell-specific dynamics (Martí et al., 2013).

NO Modulates Short-Term Ca^{2+} Responses in Arabidopsis

cADPR previously has been suggested to be involved in the NO signaling pathway in plants (Garcia-Mata et al., 2003; Lamotte et al., 2006; Zhang and Li, 2006), but measurements of NO regulation of ADPR cyclase activity and [cADPR] have not been reported. By measuring ADPR cyclase and cADPR levels, it has been possible to observe that the elevation of cADPR in response to NO is transitory (Fig. 5) and that $[Ca^{2+}]_{cyt}$ returns rapidly to resting in the absence of cADPR synthesis (Fig. 2E). We conclude that cADPR-dependent NO-regulated $[Ca^{2+}]_{cyt}$ signaling is most likely involved in shorter term responses that might occur in response to plant-pathogen interactions, symbiotic events, or hormones (Mur et al., 2013). If these rapid, short-term NO-mediated increases in $[Ca^{2+}]_{cyt}$ are involved in longer term signaling, such as the photoperiodic regulation of flowering, they are likely to be very early in the signaling cascade.

It is not known how NO regulates ADPR cyclase activity, but we have shown this to occur in a cell-free extract; therefore, it is reasonable to suspect that the effect could be direct, through a mechanism such as nitrosylation. In mammals and sea urchins, NO increases the activity of ADPR cyclase through guanylate cyclase- and cGMP-dependent pathways (Galione, 1993, 1994). There are many possible sources of NO in plants, including enzymatic and nonenzymatic (Bethke et al., 2004; Crawford, 2006). One of the most established sources of NO in plants is nitrate reductase, which usually converts NO^{3-} into NO^{2-} but also may convert NO^{2-} into NO in anaerobic conditions and when NO^{2-} levels are high (Yamasaki et al., 1999; Rockel et al., 2002; Meyer et al., 2005; Crawford, 2006). Our data do not distinguish which of those are responsible for regulating ADPR cyclase, but we do demonstrate that high endogenous levels of NO, such

as those achieved in *cml24-4* mutants, are capable of increasing ADPR cyclase activity. Our identification of NO-regulated ADPR cyclase activity fills a gap in the NO signal transduction chain. There are likely to be additional regulators of ADPR cyclase activity, because the NO-induced cADPR increase was transient, contrasting with ABA-evoked cADPR increases, which were sustained for at least 1 h (Sánchez et al., 2004).

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Measurement of $[Ca^{2+}]_{cyt}$

Experiments were performed with Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0, except where stated. Seeds were grown and $[Ca^{2+}]_{cyt}$ was measured using aequorin in plants carrying *CaMV35S:APOAEQUORIN* as described by Martí et al. (2013). Seeds were sown in petri dishes and stratified in the dark at 4°C for 2 to 3 d. Petri dishes were then transferred to a growth cabinet (12 h of light/12 h of dark, 20°C, 50–60 μmol m^{-2} s^{-1} irradiance) for 7 to 12 d for $[Ca^{2+}]_{cyt}$ measurement, 3 weeks for measuring [cADPR], and 4 to 5 weeks for ADPR cyclase activity measurement.

Measurement of ADPR Cyclase Activity in Protein Extracts

Preparation of Soluble Protein Extracts

Five to 10 g of whole rosette tissue, excluding roots, of 4- to 5-week-old Arabidopsis plants was homogenized using a pestle and mortar at 4°C in solution A (340 mM Glc [Fisher Scientific], 20 mM HEPES [Sigma], 1 mM MgCl$_2$ [BDH Laboratory Supplies], 50 g mL^{-1} soybean trypsin inhibitor [Sigma], 10 μg mL^{-1} leupeptin [Sigma], and 10 μg mL^{-1} aprotinin [Sigma], pH 7.2, 3 mL g^{-1} fresh weight). The homogenate was filtered through two layers of Miracloth (Calbiochem), and the resulting filtrate was centrifuged at 2,000g for 5 min at 4°C to remove unbroken cell debris, tissues. etc. The supernatant was transferred into a 15-mL falcon tube and centrifuged at 12,000g for 15 min at 4°C (Beckman Coulter Avanti J-26XP centrifuge). After centrifugation, the supernatant was collected carefully and run through the PD-10 desalting column (GE Healthcare) according to the manufacturer's protocols. Protein content was estimated with a protein assay kit (Bio-Rad Laboratories) using bovine serum albumin (New England Biolabs) as a standard.

NGD/NHD Assays of ADPR Cyclase Activity Using a Luminescence Spectrometer

A total of 145 μg of protein from Col-0 plants was taken in 1,200 μL of solution A (pH 7.2) in quartz cuvettes, and fluorescence intensity was measured for every minute up to 10 min at 21°C using a luminescence spectrometer (Perkin Elmer LS 55) set with the excitation wavelength at 300 nm and emission wavelength at 410 nm. After 10 min, 60 μL of 4 mM NGD (prepared in solution A, final concentration of 200 μM; Sigma) was added to reactions, and the resultant fluorescence intensity was measured for another 10 to 15 min. Additionally, fluorescence intensity was measured for every minute up to 10 to 15 min at 21°C for 1,200-μL reactions in solution A (pH 7.2) containing 145 μg of total protein, 145 μg of total protein + 200 μM NAD (Sigma), 145 μg of total protein + 200 μM NGD, and 145 μg of boiled protein (100°C; 10–15 min) + 200 μM NGD. For the NHD assay, 200 μM NHD (prepared in solution A, pH 7.2; Sigma) was used in place of NGD.

To test the effect of NO on ADPR cyclase activity in the protein extracts, 4- to 5-week-old Col-0, *cml24-4*, or *cml23-2 cml24-4* plants were incubated in the presence or absence of 300 μM SNAP (Calbiochem) or 300 μM SNAP and 300 μM cPTIO (Sigma) for 40 to 50 min, separately. Alternatively, protein extracts of untreated Col-0 plants were incubated with 300 μM SNAP or 300 μM SNAP and 300 μM cPTIO for 40 to 50 min, separately. Total protein extract of Col-0 plants (4–5 weeks old) was incubated with 50 μM ABA for 1 h.

Reverse Cyclase Assay for [cADPR] Measurement

cADPR Isolation and cADPR Purification

Plants were dosed with 150 μM SNAP or 0.5% methanol by flooding for 1 min, after which all the liquid was taken out. Plants were harvested before

dosing (0 min) and 5, 10, 30, and 60 min after. Only the aerial parts were harvested. Plants were pooled, frozen in liquid nitrogen, and stored at −80°C. Frozen samples were finely ground in liquid nitrogen. About 2 mg of frozen material was thawed and vortexed in 250 μL of ice-chilled HPLC-water (about 4°C; Fisher Scientific). Protein quantification was performed on 25 μL of the sample by Bradford assay. In order to precipitate proteins, 25 μL of 7 M perchloric acid (Sigma) was added to the samples and vortexed. One milliliter of ice-chilled 3:1 mix of 1,1,2-trichlorotrifluoroethane:tri-N-octylamine (Sigma) was added to separate cADPR from the rest of the plant extract. The mixture was vortexed and kept on ice until the precipitation of perchloric acid. Samples were centrifuged at 4°C for 10 min at 1,500g. After centrifugation, the samples had two phases separated by a white film. The aqueous phase (upper) was taken off, and 1 M NaPO$_4$ buffer (pH 8) was added to a final concentration of 20 mM NaPO$_4$. Contaminating nucleotides were removed from the isolated aqueous phase by enzymatic hydrolysis. Nucleotide pyrophosphatase (EC 3.6.1.9; Sigma) at 0.44 μg mL^{-1}, 1.25 μg mL^{-1} alkaline phosphatase (EC 3.1.3.1; isolated from bovine intestinal mucosa; Sigma), and 0.06 μg mL^{-1} NADase (EC 3.2.2.5; isolated from porcine brain; Sigma) were added to the samples and incubated overnight at 37°C. After incubation, the enzymes were separated from the extract with 3,000 molecular weight cutoff filters (500 μL; Millipore) and spun at 4°C for 30 min at 13,000g. The final extract was diluted 1:1 with 200 mM phosphate buffer to a final concentration of 100 mM NaPO$_4$.

Fluorescence-Based Cycling Assay

The cycling assay is based on a cycle of enzymatic conversions (Graeff and Lee, 2002). cADPR is first converted to NAD by ADPR cyclase (EC 3.2.2.5; from the marine sponge *Axinella polypoides*; a gift from E. Zocchi and Dr. S. Bruzzone, Universita di Genova) in the presence of high amounts of nicotinamide (Sigma). Next, alcohol dehydrogenase (EC 1.1.1.1; extracted from *Saccharomyces cerevisiae*; Sigma) converts ethanol and NAD into acetaldehyde and NADH. Finally, diaphorase (EC 1.8.1.4; extracted from *Clostridium kluyveri*; Sigma) converts NADH and resazurin (Sigma) into NAD and resorufin, a fluorescent substance that can be detected by a multifunctional microplate reader (FluoStar OPTIMA; BMG LabTech). To each well of black 96-well plates, 100 μL of sample was added. First, 50 μL of assay reagent (30 mM nicotinamide and 0.3 μg mL^{-1} ADPR cyclase in 100 mM phosphate buffer, pH 8) was added and left for 15 min, then 100 μL of cycling reagent (10 mM nicotinamide, 20 μM resazurin, 10 μM riboflavin 5-monophosphate [Sigma], 100 μg mL^{-1} alcohol dehydrogenase, 10 μg mL^{-1} diaphorase, and 2% ethanol in 100 mM NaPO$_4$ buffer, pH 8) was added to each well, and fluorescence was measured at 30°C every 5 min for 8 h at 540 ± 5 nm excitation and 590 ± 5 nm emission. Diaphorase is frequently contaminated with nucleotides, so the enzyme was purified by incubation in 2% activated charcoal in 37°C for 30 min followed by centrifugation at 5,000g for 5 min. cADPR levels were estimated using 2, 5, 10, 50, 100, and 500 nM cADPR (Calbiochem) as standards. As the cADPR isolation and purification steps in the samples might lead to cADPR losses, those steps also were performed in the cADPR standards.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Preparation of the *A. californica* standard curve.

Supplemental Figure S2. ADPR cyclase activity in response to nicotinamide.

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