Cyclic adenosine diphosphoribose (cADPR), a metabolite of NAD, appears to modulate changes in intracellular free Ca\(^{2+}\) levels by activation of ryanodine-sensitive Ca\(^{2+}\) channels. We report here that an ADPR cyclase purified from Aplysia californica readily catalyzes the conversion of NADP to 2'-phospho-cyclic adenosine diphosphoribose ([2'-P]-cADPR), cyclized at N-1 of the adenosine moiety. An enzyme from canine spleen previously shown to contain NAD glycohydrolase, ADPR cyclase, and ADPR hydrolase activities also utilized NADP and 2'-P-cADPR as substrates. The apparent \(K_{m}\) value for NADP was 1.6 \(\mu M\) compared with 9.9 \(\mu M\) for NAD, and the \(V_{\text{max}}\) with NADP was twice that with NAD, indicating that 2'-P-cADPR is a likely metabolite in mammalian cells. 2'-P-cADPR was as active as cADPR in eliciting Ca\(^{2+}\) release from rat brain microsomes, but was unable to elicit Ca\(^{2+}\) release following conversion to 2'-P-cADPR by the action of canine spleen NAD glycohydrolase. 2'-P-cADPR and 1-o-myoinositol 1,4,5-trisphosphate (IP\(_3\)) appear to act by distinct mechanisms as microsomes desensitized to IP\(_3\) still released Ca\(^{2+}\) in response to 2'-P-cADPR and vice versa. Also, inhibition of IP\(_3\)-induced Ca\(^{2+}\) release by heparin had no effect on release by 2'-P-cADPR. Both 2'-P-cADPR and cADPR appear to act by a similar mechanism based on similar kinetics of Ca\(^{2+}\) release, similar dose-response curves, cross-desensitization, and partial inhibition of release by procaine. The results of this study suggest that 2'-P-cADPR may function as a new component of Ca\(^{2+}\) signaling and a possible link between NADP metabolism and Ca\(^{2+}\) homeostasis.

Rapid changes in the cytosolic levels of free Ca\(^{2+}\), termed Ca\(^{2+}\) signaling, are involved in the regulation of diverse cellular events including fertilization, muscle contraction, secretion, and proliferation (1, 2). Often, Ca\(^{2+}\) signaling is initiated by the release of internal Ca\(^{2+}\) stores into the cytosolic compartment when endogenous second messengers activate membrane Ca\(^{2+}\) channels. Two distinct families of Ca\(^{2+}\) channels have been recognized to play key roles in intracellular Ca\(^{2+}\) mobilization in many cell types. One family consists of channels sensitive to IP\(_3\), a second messenger generated from cellular phospholipids in response to numerous hormones and neurotransmitters (1, 3, 4). A second family is composed of the ryanodine-sensitive Ca\(^{2+}\) channels (5, 6). One possible second messenger of ryanodine-sensitive channels is Ca\(^{2+}\) itself, in a process termed Ca\(^{2+}\)-induced Ca\(^{2+}\) release (6). Recently, cADPR, a naturally occurring metabolite of NAD (7, 8), has been implicated as a second messenger of ryanodine-sensitive channels. In sea urchin egg microsomes, cADPR is a potent mediator of Ca\(^{2+}\) release, and its action is cross-potentiated by agents known to affect ryanodine-sensitive Ca\(^{2+}\) channels (9–13). In mammals, cADPR has been shown to elicit Ca\(^{2+}\) release from microsomes isolated from several different tissues (14–18) and permeabilized cells (19, 20).

While cADPR appears to be a link between NAD metabolism and Ca\(^{2+}\) homeostasis, the metabolic signals that regulate cADPR synthesis and/or degradation are unknown, and our knowledge of cADPR metabolic enzymes is limited. An ADPR cyclase that catalyzes the stoichiometric conversion of NAD to cADPR has been isolated from the marine mollusk Aplysia californica (21, 22). In mammals, cADPR appears to be metabolized by multifunctional enzymes that contain ADPR cyclase, ADPR hydrolase, and NAD glycohydrolase activities (23–26). While it is not yet clear if all mammalian NAD glycohydrolases are involved in cADPR metabolism, many of these enzymes effectively use both NAD and NADP as substrates (27–33), raising the possibility that they may catalyze the formation of a cyclic nucleotide from NADP. We report here that the Aplysia ADPR cyclase, an enzyme with strong sequence homology to several mammalian NAD glycohydrolases, catalyzes the conversion of NADP to 2'-P-cADPR. In addition, a multifunctional canine spleen NAD glycohydrolase (23) containing ADPR cyclase and ADPR hydrolase activities displays a kinetic preference for NADP over NAD and utilizes 2'-P-cADPR as a substrate, indicating that 2'-P-cADPR is a likely metabolite in mammalian cells. We also report that 2'-P-cADPR is as active as cADPR in eliciting Ca\(^{2+}\) release from rat brain microsomes. Together with recent reports that another possible metabolite of NADP causes Ca\(^{2+}\) mobilization (34, 35), the results described here raise the possibility that NADP metabolism may be linked to Ca\(^{2+}\) signaling.

**MATERIALS AND METHODS**

Activity of Aplysia ADPR Cyclase and Canine Spleen NAD Glycohydrolase on NAD and NADP—ADPR cyclase was purified from A. californica as described by Hellmich and Strumwasser (21), and canine spleen NAD glycohydrolase was purified as described by Kim et al. (23). Both enzymes were judged to be homogeneous when analyzed by SDS-polyacrylamide gel electrophoresis. Both enzymes efficiently release nicotinamide from NAD, but the primary product of the Aplysia enzyme is cADPR, while the canine spleen and other mammalian enzymes release nicotinic acid; TAPS, 3-(tris(hydroxymethyl)methyl)aminopropanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; NAADP, nicotinic acid-adenine dinucleotide phosphate.

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1 The abbreviations used are: IP\(_3\), 1-o-myoinositol 1,4,5-trisphosphate; cADPR, cyclic adenosine diphosphoribose; ADPR, adenosine diphosphoribose; 2'-P-cADPR, 2'-phospho-cyclic adenosine diphosphoribose; 2'-P-ADPR, 2'-phosphoadenosine diphosphoribose; HPLC, high performance liquid chromatography; MES, 2-(N-morpholino)ethanesulfonic acid; TAPS, 3-(tris(hydroxymethyl)methyl)aminopropanesulfonic acid; CAPS, 3-(cyclohexylamino)propanesulfonic acid; NAADP, nicotinic acid-adenine dinucleotide phosphate.
(24–26) release cADPR very poorly, and thus, the primary product is ADPR. To compare the relative enzyme activity with NAD and NADP, the enzymes were assayed for release of nicotinamide. Reaction mixtures containing 20 mM HEPES, pH 7.2, 0.1% Triton X-100, and varying concentrations of [2H]NAD or [3H]NADP containing radiolabeled in the nicotinamide moiety were incubated with the enzymes in a total volume of 50 μl at 37°C. Incubation was terminated by adding 50 μl of 0.1% SDS, and samples were applied to 0.5-ml columns of Bio-Rad AG 1-X2 anion-exchange resin. The columns were subsequently washed with 5 ml of deionized water. The amount of [3H]nicotinamide in the flow-through and wash fractions was determined by liquid scintillation counting.

Aplysia CNS NAD Glycohydrolase on 2'-P-ADPR—To assay for conversion of 2'-P-ADPR to free 2'-P-ADPR, reaction mixtures containing varying concentrations of [3H]2'-P-ADPR in 20 mM HEPES, pH 7.2, 0.1% Triton X-100, and canine spleen NAD glycohydrolase in a total volume of 50 μl were incubated at 37°C for 30 min. The incubation was terminated by addition of 50 μl of 0.2 M NaOH followed by 50 μl of 0.16 M HCl and 20 μl Tris free base. The [3H]2'-P-ADPR produced was converted to adenosine by addition of 10 μl of 100 mM MgCl2 and 20 μl containing 0.5 unit of snake venom phosphodiesterase (Worthington) and 0.5 unit of bacterial alkaline phosphatase (Sigma). After incubation at 37°C for 30 min, incubation mixtures were diluted to 1.0 ml with 0.1% SDS and applied to 0.5-ml columns of Bio-Rad AG 1-X2 anion-exchange resin. The columns were subsequently washed with 5 ml of deionized water. The amount of [3H]adenosine in the flow-through and wash fractions was determined by liquid scintillation counting.

Enzymatic Synthesis and Purification of 2'-P-ADPR—An immobilized Aplysia ADPR cyclase was prepared by coupling the purified enzyme to an agarose g. Tresyl chloride-activated agarose (0.5 g; Tropix, Bedford, MA) was immersed overnight in 5 ml of 0.2 M sodium bicarbonate, pH 8.2, then washed with 10 ml of water. The immobilized enzyme was transferred to a 0.8 ml bed volume. For use, 250 mM sodium bicarbonate, pH 8.2, was applied to the immobilized enzyme and allowed to pass through the column. The column was then washed with 20 ml of buffer A followed by 5 ml of deionized water. Elution was performed using 5 ml of 100 mM ammonium formate buffer, pH 4.0. The eluate was applied to a 10 × 270-mm Dynamax preparative reversed-phase HPLC column (Rainin Instrument Co. Inc.) with isocratic elution with 0.1% TFA at a flow rate of 2 ml/min. The 2'-P-ADPR peak was collected and lyophilized overnight. To eliminate residual trifluoroacetic acid, the preparation was redissolved in deionized water and subjected to two additional cycles of lyophilization. The final sample was reconstituted in deionized water and stored at −20°C. The concentration of 2'-P-ADPR in stock solutions was determined by absorbance at 254 nm using an extinction coefficient of 14,300 (36).

Characterization of 2'-P-ADPR—For treatment with alkaline phosphatase, 600 nmol of 2'-P-ADPR in a total volume of 0.1 ml of 20 mM HEPES, pH 7.2, was incubated with 0.2 unit of bacterial alkaline phosphatase at 37°C for 1 h. Aliquots were removed and analyzed on a 4.6 × 250-mm Partisil-10 strong anion-exchange HPLC column (Whatman) with 50 mM potassium phosphate buffer, pH 6.0, as the mobile phase at a flow rate of 1 ml/min. Treatment with canine spleen NAD glycohydrolase (23) was done by incubating 15 nmol of 2'-P-ADPR in 0.05 ml of 20 mM HEPES, pH 7.2, and 0.1% Triton X-100 with 0.5 μg of purified enzyme at 37°C for 20 min. HPLC analysis was performed using the Partisil-10 strong anion-exchange HPLC system described above. Treatment with snake venom phosphodiesterase was done by incubating 25 nmol of 2'-P-ADPR in 0.1 ml of 20 mM HEPES, pH 7.2, 20 μg snake venom phosphodiesterase, and 5 μM MgCl2, with 0.2 unit of snake venom phosphodiesterase at 37°C for 30 min. Aliquots were taken from the reaction mixture and subjected to reversed-phase HPLC with iso-
NADP was passed through a column of immobilized enzyme as shown in Fig. 1B. Passage through the column resulted in the disappearance of ~90% of NADP and the appearance of nicotinamide and unidentified material eluting at 4 min. The material eluting at 4 min was subsequently purified as described under "Materials and Methods." The HPLC chromatograms of the NADP solution before and after passage through the ADPR cyclase column are shown in A and B, respectively. The material eluting at 4 min was subsequently purified as described under "Materials and Methods," and an aliquot of the purified material was analyzed by reversed-phase HPLC as shown in C.

Characterization of Material Produced from NADP—The material derived from passage of NADP through the Aplysia ADP cyclase column was characterized by both enzymatic and spectral methods. When subjected to anion-exchange HPLC (Fig. 2A), ~95% of the UV-absorbing material eluted at 21 min, with a trace amount of material eluting at 8 min. The elution time of 21 min did not correspond to the elution times of cADPR (5 min), ADPR (7 min), and 2'-P-ADPR (43 min). Incubation with alkaline phosphatase resulted in a near quantitative conversion of the material eluting at 21 min to material that coeluted with cADPR, while the material that eluted at 8 min was unaffected by the phosphatase treatment (Fig. 2B). Incubation with purified canine spleen NAD glycohydrolase, an enzyme previously shown to catalyze the hydrolysis of cADPR to ADPR (23), resulted in a near quantitative conversion to material that coeluted with 2'-P-ADPR (data not shown). Incubation with snake venom phosphodiesterase, which does not act on cADPR (23), did not affect the chromatographic properties of the material, while in a positive control, ADPR was completely converted to material that comigrated with AMP (data not shown). In total, the enzymatic characterizations indicated that the material was a cyclic form of 2'-P-ADPR.

Further information was obtained from 1H NMR spectroscopy (Fig. 3). The 1H NMR spectrum was very similar to that of cADPR (36–38). The spectrum also indicated that the material was primarily a single compound. A unique similarity to the spectrum of cADPR was the presence of two sets of chemical shifts between 6.0 and 6.2 ppm, which correspond to the anomeric protons of both ribose moieties. The chemical shifts at this frequency indicate that both ribose anomeric carbon atoms are bound to nitrogen, i.e. that the nucleotide is cyclic. A second unique similarity was an isolated signal with a chemical shift between 5.4 and 5.5 ppm. In cADPR, this signal is due to the ribose 2'-proton, which is shifted far downfield relative to the other ribose protons (36–38). This signal appears as a well resolved triplet, while a broad singlet was seen for the cyclic form of 2'-P-ADPR. This difference can be attributed to the presence of a phosphate esterified to the 2'-carbon, with a resulting phosphorus-proton coupling.

The position of cyclization was studied by obtaining UV absorption spectra as a function of pH. Fig. 4 shows spectra...
obtained at pH values of 5.0, 9.0, and 11.0. With increasing pH, the compound displayed a hyperchromic effect at 260 nm, a more pronounced shoulder at 267 nm, and a markedly increased absorbance in the region at 280–310 nm. These pH-dependent spectral changes are unique to cADPR and other adenosine nucleotides substituted at N-1 of the adenine ring (41), indicating that N-1 was the position of cyclization in 2'-P-cADPR. The spectral changes in the region at 280–310 nm are due to dissociation of an adenine ring proton (41). Fig. 4 (inset) shows a plot of the absorbance at 300 nm as a function of pH. From these data, a pKₐ of ~9.0 was determined for 2'-P-cADPR. The corresponding pKₐ value for cADPR is ~8.2 (41). The higher pKₐ value of 9.0 can be attributed to the presence of the additional phosphate group present in the molecule. Taken together, the data from both enzymatic and spectral characterizations demonstrate that the compound generated from NADP by the action of immobilized A. californica ADPR cyclase is 2'-P-cADPR, cyclized at N-1 of the adenine moiety (Fig. 5).

Utilization of NADP and 2'-P-cADPR by Canine Spleen NAD Glycohydrolase—Our laboratory has described previously the isolation and characterization from canine spleen of a multifunctional enzyme that contains NAD glycohydrolase, ADPR cyclase, and cADPR hydrolase activities (23). This enzyme uses either NAD or cADPR as substrate. Similar enzymes have been described in other mammalian cells (24–26), indicating that multifunctional NAD glycohydrolases may be the rule for cADPR metabolism in mammalian cells. To address the question as to whether 2'-P-cADPR was a likely metabolite in mammalian cells, we determined if the canine spleen enzyme would utilize NADP and 2'-P-cADPR as substrates. Fig. 6A shows that the enzyme utilized NADP as a substrate. From three separate experiments, an apparent Kₘ value for NADP of 1.6 ± 0.2 μM was determined. The corresponding Kₘ value for NAD was determined with the same preparation of enzyme, and the value obtained was not significantly different from the value of 9.9 μM that we had previously reported (23). The relative Vₘₐₓ values for NADP and NAD were also determined.
and the $V_{\text{max}}$ for NADP was $\sim 200\%$ of that observed for NAD. Also, competition experiments with NAD and NADP indicated that NAD was a competitive inhibitor of NADP and vice versa (data not shown). Accordingly, the specificity constant value $k_{\text{cat}}/K_m$ for NADP is 12 times greater than the value for NAD, indicating that the NADP is a more preferred substrate than NAD. The canine spleen and other mammalian enzymes release cADPR very poorly; thus, the primary product released from the enzyme when NAD is used as a substrate is ADPR rather than cADPR (23–26). Likewise, the primary product released from the canine spleen enzyme when NADP was used as a substrate was $2^\prime$-P-ADPR rather than $2^\prime$-P-cADPR (data not shown). Fig. 6B shows that $2^\prime$-P-cADPR was also a substrate for the enzyme. From three separate experiments, the apparent $K_m$ value for $2^\prime$-P-cADPR was $140 \pm 14 \mu M$. The corresponding $K_m$ value for cADPR was also determined and was similar to the value of $46 \mu M$ reported previously (23). The $V_{\text{max}}$ for $2^\prime$-P-cADPR was $\sim 10\%$ of that observed for cADPR. In total, our results show that the canine spleen NAD glycohydrolase functions more effectively with NADP than with NAD as a substrate, but less effectively with $2^\prime$-P-cADPR than with cADPR. These data indicate that the conversion of NAD to $2^\prime$-P-cADPR is likely to occur in vivo.

**Fig. 6. Activity of canine spleen NAD glycohydrolase with NADP and $2^\prime$-P-cADPR as substrates.** The activity of canine spleen NAD glycohydrolase (23) with NADP (A) or $2^\prime$-P-cADPR (B) as substrate was determined as described under "Materials and Methods." Representative Lineweaver-Burk plots are shown. From three separate experiments, apparent $K_m$ values of $1.6 \pm 0.2 \mu M$ for NADP and $140 \pm 14 \mu M$ for $2^\prime$-P-cADPR were determined.

**Fig. 7. Comparison of Ca$^{2+}$ mobilizing characteristics of $2^\prime$-P-cADPR, cADPR, IP$_3$, $2^\prime$-P-ADPR, and ADPR from rat brain microsomes.** A, C, and E show the microsomal response to additions of $10 \mu M$ $2^\prime$-P-cADPR, cADPR, and IP$_3$, respectively. Additions of $50 \mu M$ $2^\prime$-P-ADPR and ADPR are shown in B and D, respectively.

Ca$^{2+}$ Mobilizing Activity of $2^\prime$-P-cADPR—The Ca$^{2+}$ mobilizing activity of $2^\prime$-P-cADPR was studied using rat brain microsomes. Fig. 7A shows that addition of $10 \mu M$ $2^\prime$-P-cADPR resulted in Ca$^{2+}$ release from the microsomes. The pattern of Ca$^{2+}$ release appeared to be biphasic, with an initial rapid release followed by a sustained but slower rate of release. A very similar pattern of Ca$^{2+}$ release was observed by addition of $10 \mu M$ cADPR (Fig. 7C). This similarity raised a concern that the Ca$^{2+}$ release observed following addition of $2^\prime$-P-cADPR was the result of conversion of $2^\prime$-P-cADPR to cADPR by the rat brain microsomes, with the observed Ca$^{2+}$ release being due to cADPR rather than to $2^\prime$-P-cADPR. To examine this possibility, samples were removed from incubation mixtures such as those shown in Fig. 7 and subjected to anion-exchange HPLC analyses. There was no detectable decrease in $2^\prime$-P-cADPR, even after a prolonged incubation, indicating that little or no conversion of $2^\prime$-P-cADPR to cADPR occurred under these conditions (data not shown).

The Ca$^{2+}$ release elicited by both $2^\prime$-P-cADPR and cADPR was dose-dependent (Fig. 8). EC$_{50}$ values of $1.3 \mu M$ for $2^\prime$-P-cADPR and $1.5 \mu M$ for cADPR were determined. The Ca$^{2+}$ release was specific for the cyclic nucleotides as addition of $50 \mu M$ $2^\prime$-P-ADPR (Fig. 7B) and ADPR (Fig. 7D) did not result in
detectable Ca\(^{2+}\) release. Also shown is Ca\(^{2+}\) release following addition of 10 \(\mu\)M IP\(_3\) (Fig. 7E). In contrast to the patterns observed with 2'-P-cADPR and cADPR, the IP\(_3\) response showed a rapid phase of Ca\(^{2+}\) release followed by rapid re-uptake of Ca\(^{2+}\) into the microsomes.

Current evidence indicates that cADPR and IP\(_3\) elicit Ca\(^{2+}\) release through distinct mechanisms (10). To determine if the mechanism of Ca\(^{2+}\) release induced by 2'-9-P-cADPR was also distinct from the IP\(_3\) system, cross-desensitization and inhibition studies were done. Fig. 9A shows that a second addition of IP\(_3\) resulted in Ca\(^{2+}\) release that was only 4% of the first addition, consistent with desensitization of the IP\(_3\)-sensitive release mechanism. However, a subsequent addition of 2'-P-cADPR elicited approximately the same amount of Ca\(^{2+}\) release observed without prior additions of IP\(_3\). Fig. 9B shows that a second addition of 2'-P-cADPR resulted in Ca\(^{2+}\) release that was 29% of the first addition, indicating a partial desensitization of the 2'-P-cADPR release mechanism. In this case, a subsequent addition of IP\(_3\) still resulted in Ca\(^{2+}\) release that was approximately the same as observed without prior additions of 2'-P-cADPR (Fig. 9B). The relationship between 2'-P-cADPR and IP\(_3\) was examined further by the use of heparin, a potent inhibitor of IP\(_3\)-sensitive Ca\(^{2+}\) channels (1). Pretreatment with 600 \(\mu\)g/ml heparin completely abolished the action of IP\(_3\), but the Ca\(^{2+}\) release activity of 2'-P-cADPR was unaffected (data not shown). In total, these data provide evidence that the mechanism of 2'-P-cADPR-induced Ca\(^{2+}\) release is distinct from the IP\(_3\) pathway.

Evidence that 2'-P-cADPR and cADPR were acting via a similar mechanism was obtained by examination of the effects of 2'-P-cADPR on cADPR-induced Ca\(^{2+}\) release and vice versa. In the experiment shown in Fig. 10A, a second addition of 20 \(\mu\)M 2'-P-cADPR resulted in Ca\(^{2+}\) release that was 24% of the first addition. The subsequent addition of cADPR resulted in Ca\(^{2+}\) release that was 32% of a control without 2'-P-cADPR pretreatment, indicating that the 2'-P-cADPR release mechanism was cross-desensitized to that of cADPR, even though the release mechanism was only partially desensitized. A similar result was observed when successive additions of cADPR were followed by addition of 2'-P-cADPR (Fig. 10B). Partial desensitization of Ca\(^{2+}\) release was also observed following addition of subsaturating amounts of 2'-P-cADPR. For example, when the microsomes were treated with successive additions of 1 \(\mu\)M 2'-P-cADPR, the response to the second, third, and fourth additions was reduced to 25–35% of the initial addition (data not shown). In the same experiment, when 1 \(\mu\)M cADPR was added following the four additions of 2'-P-cADPR, Ca\(^{2+}\) release was 25% of a control without 2'-P-cADPR pretreatment, again indicating cross-desensitization between the 2'-P-cADPR and cADPR mechanisms.

Evidence that both 2'-P-cADPR and cADPR were operating through a ryanodine-sensitive Ca\(^{2+}\) channel was obtained by examining the effect of procaine, a partial antagonist of ryan-
When the microsomes were pretreated with 5 mM procaine, the Ca\(^{2+}\) release induced by 1 μM cADPR or 2'-P-cADPR was inhibited by 45 and 50%, respectively, relative to the controls (Fig. 11).

**DISCUSSION**

Studies with cADPR have provided a link between NAD metabolism and regulation of Ca\(^{2+}\) signaling (10). The results described here, together with recent reports that another possible NADP metabolite, NAADP, causes Ca\(^{2+}\) release in sea urchin egg microsomes (34, 35), suggest a possible link between NADP metabolism and Ca\(^{2+}\) signaling. As shown here, the Aplysia ADPR cyclase utilizes NAD and NADP with very similar efficiency to generate cyclic nucleotides (Fig. 1). In mammalian cells, the only enzymes that have been demonstrated to metabolize cADPR are NAD glycohydrolases that catalyze both the synthesis of cADPR from NAD and the hydrolysis of cADPR to ADPR (23–26). Our results with the canine spleen enzyme using NADP as a substrate indicate that the conversion of NADP to 2'-P-cADPR in vivo is likely. The \(K_m\) value for the enzyme for NADP is well below estimated cellular concentrations of NADP (42), and the enzyme displays a kinetic preference for NADP over NAD as reflected by a significantly higher ratio of \(k_{cat}/K_m\) for NADP compared with NAD. Evidence that the multifunctional canine spleen enzyme uses the same mechanism (23) with NADP as with NAD is supported by the observation that NAD and NADP are competitive substrates and that the enzyme also uses both cADPR and 2'-P-cADPR as substrates. While it remains to be determined if NADP is converted to 2'-P-cADPR in mammalian cells, the efficiency with which the canine spleen enzyme uses NADP warrants a search for 2'-P-cADPR in vivo.

The maintenance of Ca\(^{2+}\) homeostasis requires the precise regulation of cytosolic free Ca\(^{2+}\) levels. These levels are in turn controlled by membrane Ca\(^{2+}\) channels through which Ca\(^{2+}\) moves between different intracellular compartments and between intracellular and extracellular compartments. The elevation of cytosolic free Ca\(^{2+}\) levels is often initiated by activation of members of one or both of two different families of intracellular Ca\(^{2+}\) channels, the IP\(_3\)-sensitive channels (1, 3, 4) and the ryanodine-sensitive channels (5, 6). Studies of sea urchin egg microsomes indicate that cADPR modulates ryanodine-sensitive Ca\(^{2+}\) channels (9–13). Although less well characterized, cADPR also appears to act on ryanodine-sensitive Ca\(^{2+}\) channels in mammalian cell microsomes (14, 15). The results presented here indicate that 2'-P-cADPR, similar to cADPR, causes Ca\(^{2+}\) release by a mechanism distinct from IP\(_3\).

Microsomes desensitized to further addition of IP\(_3\) could still release Ca\(^{2+}\) in response to 2'-P-cADPR and vice versa (Fig. 9). Also, levels of heparin that completely blocked release by IP\(_3\) did not affect Ca\(^{2+}\) release by 2'-P-cADPR.

Our results also suggest that 2'-P-cADPR elicits Ca\(^{2+}\) release by a mechanism similar to that of cADPR based on the similar kinetics of Ca\(^{2+}\) release elicited by the two nucleotides (Fig. 7), the similar dose-response curves (Fig. 8), cross-desensitization (Fig. 10), and the partial inhibition of Ca\(^{2+}\) release by procaine (Fig. 11). The possibility that 2'-P-cADPR was being converted to cADPR by the brain microsomes and that the observed Ca\(^{2+}\) release was due to cADPR can be ruled out by the observations that there was no detectable conversion of 2'-P-cADPR to cADPR under the conditions used and that very similar dose-response curves of cADPR and 2'-P-cADPR were observed. The concentrations of 2'-P-cADPR and cADPR that gave half-maximal Ca\(^{2+}\) release in our study, 1.3–1.5 μM (Fig. 11).
It is interesting that the kinetics of Ca\(^{2+}\) release of 2'-P-cADPR and cADPR differ from those of IP\(_3\) in the brain microsomes. Addition of IP\(_3\) to the microsomes resulted in a rapid release followed by a rapid re-uptake of Ca\(^{2+}\), while 2'-P-cADPR and cADPR caused an initial rapid release followed by a slower but prolonged increase in Ca\(^{2+}\) (Fig. 7). Previous studies also have observed a prolonged Ca\(^{2+}\) release by cADPR in rat pituitary cells (20) and rat brain microsomes (14). The difference in kinetics suggests that the mode of Ca\(^{2+}\) channel activation caused by cADPR and 2'-P-cADPR may be fundamentally different from that of IP\(_3\). The rate of Ca\(^{2+}\) release during the prolonged phase that occurs following 2'-P-cADPR and cADPR is probably underestimated as it presumably reflects the actual rate of Ca\(^{2+}\) release minus the rate of Ca\(^{2+}\) re-uptake.

Even if cADPR and 2'-P-cADPR act on the same Ca\(^{2+}\) channels, the linkage of both NAD and NADP metabolism to Ca\(^{2+}\) signaling raises interesting metabolic possibilities as the NAD(H) and NADP(H) pools are functionally distinct. The NAD(H) pool is maintained in a highly oxidized state (42) as NAD serves as a hydride acceptor in multiple catabolic reactions and as a source of ADP-ribosyl transfer enzymes (43). In contrast, the NADP(H) pool is normally maintained in a highly reduced state to provide NADPH as a source of reducing equivalents for anabolic pathways (42). The highly reduced state of the NADP(H) pool under normal conditions is of interest that the studies of Richter and Kass (44) have revealed.

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