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Boric acid inhibits adenosine diphosphate-ribosyl cyclase non-competitively

Danny H. Kim [a], Shane Que Hee [a], Andrew J. Norris [b,c], Kym F. Faull [b], Curtis D. Eckhert [a,*]

[a] *Department of Environmental Health Sciences, Box 951772, University of California, 650 Charles E Young Dr South, Los Angeles, CA 90095-1772, USA*
[b] *Pasarow Mass Spectrometry Laboratory, Departments of Psychiatry & Biobehavioral Sciences and the Jane and Terry Semel Institute for Neuroscience and Human Behavior, University of California, 10833 Le Conte Avenue, Los Angeles, CA 90095, USA*
[c] *UCLA Johnson Comprehensive Cancer Center, University of California, 10833 Le Conte Avenue, Los Angeles, CA 90095, USA*

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Abstract

Adenosine diphosphate-ribosyl cyclase (ADP-ribosyl cyclase) is a ubiquitous enzyme in eukaryotes that converts NAD^+ to cyclic-ADP-ribose (cADPR) and nicotinamide. A quantitative assay for cADPR was developed using capillary electrophoresis to separate NAD^+, cADPR, ADP-ribose, and ADP with UV detection (254 nm). Using this assay, the apparent K_m and V_{max} for *Aplysia* ADP-ribosyl cyclase were determined to be 1.24 ± 0.05 mM and 131.8 ± 2.0 μM/min, respectively. Boric acid inhibited ADP-ribosyl cyclase non-competitively with a K_i of 40.5 ± 0.5 mM. Boric acid binding to cADPR, determined by electrospray ionization mass spectrometry, was characterized by an apparent binding constant, K_A, of 655 ± 99 L/mol at pH 10.3.

Keywords: ADP-ribosyl cyclase; Boron; Capillary electrophoresis; cADPR; NAD; Mass spectrometry; Enzyme kinetics

1. Introduction

Adenosine diphosphate-ribosyl cyclase (ADP-ribosyl cyclase) is widely distributed in nature and is expressed in over 40 different species of protists, plants, and animals [1–5]. The enzyme cyclizes NAD^+ to produce cyclic-ADP-ribose (cADPR) with the release of nicotinamide (Fig. 1) [6]. cADPR acts as a second messenger that mobilizes Ca^{2+} from the endoplasmic reticulum via activation of ryanodine receptors [7]. Three homologs of the cyclase with 30% sequence identity have now been identified [8–12]. There is a soluble cyclase present in the sea hare *Aplysia*, the membrane-bound lymphocyte antigen CD38, and another antigen BST-1. Mammalian CD38 is a multiple function cell surface molecule possessing both cyclase activity that converts NAD^+ to cADPR, and hydrolase activity that converts cADPR to ADPR. At pH 4.5, CD38 also converts $NADP^+$ to nicotinic acid adenine dinucleotide phosphate (NAADP), another second messenger that also triggers Ca^{2+} release from intracellular stores. However, these stores are different from those affected by cADPR [13].

The soluble *Aplysia* cyclase has been recombinantly produced in yeast and crystallized [14]. This enzyme only catalyzes the synthesis of cADPR. Lee [8] has proposed a catalytic model for the CD38 type based on an active site that consists of a highly conserved sequence containing 10 cysteine residues and three other critical residues: glutamate 179, which is the catalytic residue and lies in the catalytic pocket, and two tryptophan residues. The model proposes that the two tryptophan residues bind and fold the linear NAD^+ molecule, while glutamate attack releases the nicotinamide moiety. The adenine ring then reacts with the terminal ribose to form cADPR [8]. According to this model, disruption of NAD^+ folding in the active site may slow the catalytic activity of the enzyme. The CD38 type has about 25% sequence identity with the *Aplysia* type. Boron as boric acid and borate binds to NAD^+ [15] and therefore may affect the activity of ADP-ribosyl cyclase.

Boric acid has affinity for diol-containing compounds such as carbohydrates, where its strong complexation is now being

* Corresponding author. Tel.: +1 310 825 8429; fax: +1 310 825 8429.
E-mail address: ceckhert@ucla.edu (C.D. Eckhert).

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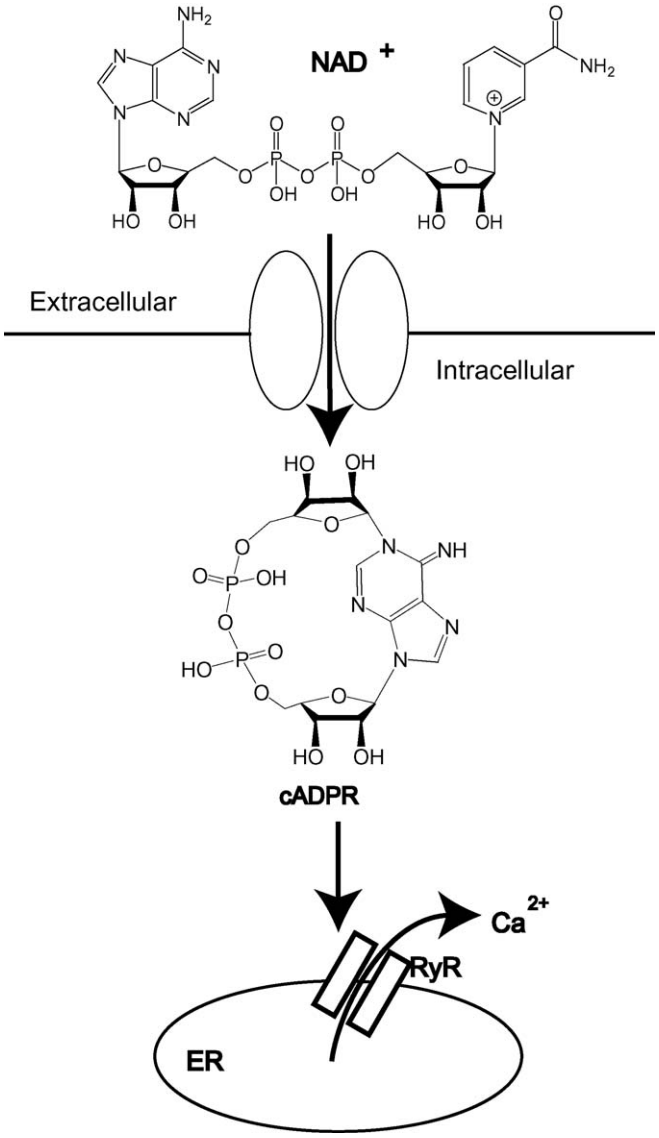


Fig. 1. Schematic view of the cADPR signaling pathway. NAD^+ is converted to cADPR, which interacts with the ryanodine receptor (RyR) through an unknown mechanism. cADPR mobilizes Ca^{2+} from endoplasmic reticulum (ER) stores by activation of RyR.

used for the fabrication of carbohydrate sensors and transporters (Eq. (1)) [16–18].

(1)

Diols are also present in the carbohydrate moieties of nucleosides and nucleotides including NAD^+, NADH, $NADP^+$, adenosine, and guanosine. Previously, we used electrospray ionization mass spectrometry (ESI-MS) to demonstrate that boric acid binds to *cis*-diols on both ribose groups on NAD^+ [15]. A comparison of 16 different nucleotides containing *cis*-diols determined that NAD^+ had highest affinity for boric acid [19]. NAD^+ affinity was about seven times greater than NADH. Migaud et al. [20] described that the ribosyl diol moiety adjacent to the nicotinamide group is important in binding NAD^+ in the active site of ADP-ribosyl cyclase. Since boric acid can bind to this diol, such binding may influence the conversion of NAD^+ to cADPR through ADP-ribosyl cyclase.

In recent years, capillary electrophoresis (CE) has emerged as a powerful separation technique to measure water-soluble metabolites, including nucleotides, for several reasons: analysis without derivatizations; simple sample preparation, short analysis time; and small sample quantity as compared to high-performance liquid chromatography (HPLC) [21,22]. On the other hand, previous assays for ADP-ribosyl cyclase mostly relied on reverse phase and anion-exchange HPLC [23–26]. In order to take advantages of this alternate separation technique, for the first time, CE was utilized to investigate the enzyme kinetics of ADP-ribosyl cyclase. The objective of the present study was to determine if boric acid binds directly to cADPR and to assess the impact of boric acid on ADP-ribosyl cyclase kinetics. The results suggest that boric acid may be involved in modulating Ca^{2+} mobilization. Ca^{2+} serves as a communicating signal that initiates a myriad of changes including control of muscle contraction and neurotransmitter release, cell migration [27], cell cycle progression [28,29], angiogenesis [30], apoptosis [31], and proliferation [32].

2. Materials and methods

2.1. Chemicals and reagents

ADP-ribosyl cyclase purified from *Aplysia californica*, nicotinamide adenine dinucleotide, oxidized form (NAD^+) (free acid), cADPR, adenosine 5′-diphosphate (ADP) (sodium salt), ADP-ribose (ADPR), boric acid, and tris(hydroxymethyl) aminomethane (Tris) were purchased from Sigma Aldrich (St. Louis, MO, USA). Enriched $^{11}B(OH)_3$ (99.27% purity) was purchased from Eagle-Picher Technologies (Quapaw, OK, USA). Ultrapure water containing <10 nM boron was used for all the experiments. Ultrapure water was prepared by ion-exchange treatment, and the boron content was checked by inductively coupled plasma mass spectrometry as previously described [33]. All other reagents and solvents were of analytical grade or higher.

2.2. Enzyme reactions

To investigate the effect of boric acid on the synthesis of cADPR, ADP-ribosyl cyclase was incubated with various concentrations of NAD^+ and boric acid in 200 mM Tris buffer (pH 7.4). Each reaction tube contained Tris (pH 7.4) and NAD^+ (0.5, 0.75, 1, 1.5, 2, 3, 4, or 5 mM) in 300 μL. Reactions were started by addition of 0.156 units of ADP-ribosyl cyclase (100 μL) and incubated at 37 °C for 0, 60, 90, and 120 s. Reactions were terminated by mixing 120 μL of the reaction mixture with 360 μL of cold 90% acetonitrile containing 97 μM ADP (73 μM final concentration) as internal standard. Each sample was centrifuged (14,000 × *g*, 4 °C, 10 min), and before analysis by CE, 200 μL was removed and added to 200 μL of 100 mM borate buffer

(pH 9.2), which was used as a background electrolytes for the electrophoretic separation of cADPR. Schmitt-Kopplin et al. [34] and Hoffstetter-Kuhn et al. [35] showed that borate buffer enhanced peak shape and changed migration time by forming a complex with the substrates. For blank and standard samples, the enzyme was denatured prior to the addition of the substrate mixture followed by the same methodology as above to determine the standard curve. For the inhibition analysis, NAD^+ was varied from 0, 0.75, 1, 1.5, 2, or 4 mM, and the boric acid concentration was varied between 0, 30, 35, and 40 mM for each NAD^+ concentration.

2.3. Capillary electrophoresis

Using an Agilent HP-CE model G1600AX (Waldbronn, Germany) CE system equipped with a diode array detector, a 50 μm i.d. × 56 cm bulbed glass capillary was pretreated with 0.1 M KOH for 10 min and then equilibrated with 100 mM borate buffer of pH 9.2 for 5 min before loading each sample. Samples were loaded by hydrostatic pressure at 50 mbar for 15 s and separations were performed at 20 °C using 20 kV producing about 24 mA of current, for 25 min duration. The effluent was monitored by absorption at 254 nm. A solution of acetone was used as an electroosmotic flow marker. One hundred and twenty microliters of standard samples containing varying amounts of cADPR (0, 12.5, 25, 50, 125, 250, and 375 μM) in the reaction mixture were mixed with 360 uL of 97 μM ADP (73 μM final concentration) in 90% cold acetonitrile and separated with CE. cADPR and ADP peak areas were computed using a program supplied with the instrument (3D-CE Chemstation, rev. A. 10.01, Waldbronn, Germany). A calibration curve was constructed by plotting the cADPR/ADP peak area ratio against amount of cADPR for the standard samples. The concentration of cADPR in the reaction samples was computed by interpolation from the standard curve.

2.4. Sample preparation for ESI-MS analysis

All solutions were prepared fresh in ultrapure water, then diluted to the desired concentration in water/acetonitrile/triethylamine (WAT, 50/50/0.2, v/v/v, pH 10.3). The borate complexes were prepared by mixing equal volumes of boric acid and cADPR solutions, yielding final concentrations of 500 and 100 μM, respectively. To determine the apparent K_A, a minimum of 12 different measurements (made on three separate occasions, each time with fresh sample preparations) were made and analyzed. The mean and standard deviation were computed.

2.5. ESI-MS

A Perkin-Elmer Sciex (Thornhill, Canada) API III triple quadrupole mass spectrometer fitted with an Ionspray™ source was tuned and calibrated in the positive ion mode as previously described [36]. Instrument resolution allowed for a 15–20% valley between the ^{13}C-containing satellites of the polypropylene glycol/NH_4^+ singly charged calibrant ion at m/z 906. For analysis of borate esterification to the nucleotides, the instrument polarity was reversed and the ion spray voltage was lowered to −3.5 kV. Samples dissolved in WAT were introduced (10 μL/injection) into a stream of the same solvent entering the ion source (10 μL/min). Spectra were collected (profile mode) while the instrument was scanning from *m/z* 520–620 (0.1 Da step size, 6 ms dwell time, 6.66 s/scan, orifice −60 V). Representative spectra were computed as the average of all the spectra accrued from each injection using instrument-supplied software (MacSpec, version 3.3 PE Sciex, Ontario, Canada).

3. Results

3.1. Michaelis–menten plot of ADP-ribosyl cyclase

CE gave clear separation of cADPR, ADP and the other nucleotides tested, with typical migration times for NAD^+, cADPR, and ADP of 13.7, 15.7, and 22.3 min, respectively. The response at 254 nm was quantitative as demonstrated by the linear standard curve ($y = 2.362x$, $r^2 = 0.999$, $p < 0.001$) across the 0–400 μM concentration range. The incubation times were adjusted such that less than 20% of the substrates were converted. The apparent K_m and V_{max} for ADP-ribosyl cyclase for NAD^+ were calculated to be 1.24 ± 0.05 mM and 131.8 ± 2.0 μM/min (r^2 of 0.999, $p < 0.001$), respectively, through nonlinear regression of the data fit to the Michaelis–Menten equation (Fig. 2). Likewise, using a Lineweaver and Burk plot, the apparent K_m and V_{max} were determined to be 1.12 ± 0.05 mM and 126.3 ± 5.3 μM/min with r^2 of 0.991, $p < 0.001$.

3.2. Inhibition of ADP-ribosyl cyclase by boric acid

With an initial NAD^+ concentration of 1 mM and boric acid concentrations between 0–40 mM, CE revealed peaks at 13.7, 15, and 22 min representing NAD^+, cADPR, and ADP, respec-

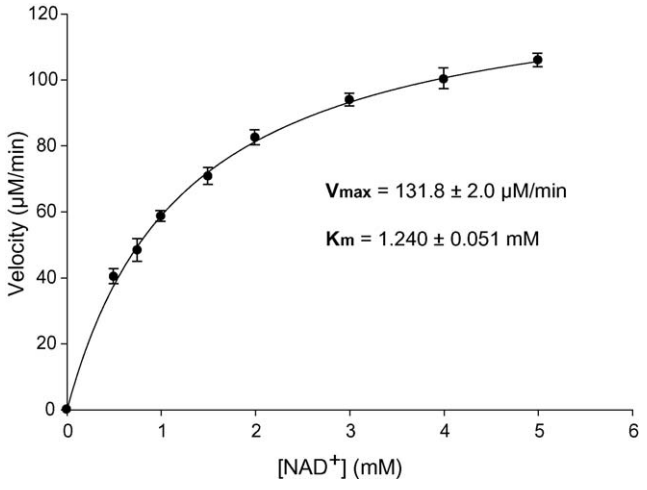


Fig. 2. Michaelis–Menten plot of ADP-ribosyl cyclase activity with NAD^+ substrate using capillary electrophoresis for separation of the reaction components and detection by absorption at 254 nm. The samples were incubated in 37 °C at pH 7.4 in Tris buffer. Nonlinear regression of the data fitted to the Michaelis–Menten equation was used to determine the K_m (1.240 ± 0.051 mM) and V_{max} (131.8 ± 2.0 μM/min).

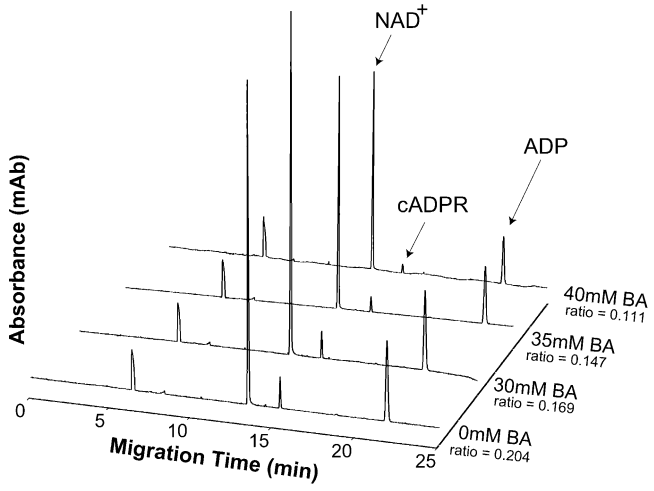


Fig. 3. Capillary electrophoretograms of the ADP-ribosyl cyclase assay in the presence of increasing boric acid concentrations. ADP was used as an internal standard. NAD^+ (1 mM) and various boric acid concentrations were reacted at 37 °C and pH 7.4 for 90 s. The area ratio of cADPR/ADP decreased as boric acid concentration increased.

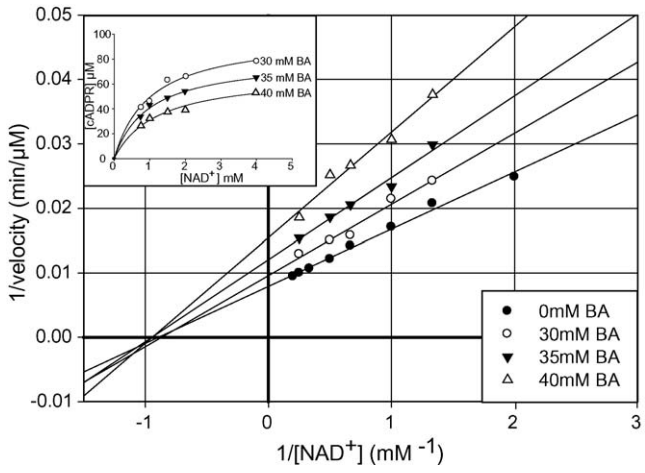


Fig. 5. Lineweaver and Burk plots reveal that the inhibition of ADP-ribosyl cyclase by boric acid (BA) is non-competitive. At the various NAD^+ concentrations, reactions occurred at 37 °C and pH 7.4. Inset: Michaelis–Menten plots at 30, 35, 40 mM BA are shown. Nonlinear regression of the data fit to the Michaelis–Menten equation revealed that K_m were similar but V_{max} decreased.

tively (Fig. 3). The ratio of cADPR to ADP decreased as boric acid concentration increased from 0 to 40 mM, showing that boric acid slowed the conversion of NAD^+ to cADPR (Fig. 4). At 40 mM, boric acid caused a 52% decrease in the activity of ADP-ribosyl cyclase.

Methyl boric acid was used as a negative control for boric acid in the inhibition of ADP-ribosyl cyclase (Fig. 4). At 40 mM, methyl boric acid had no detectable effect on the rate of cADPR formation (Fig. 4). In fact, methyl boric acid partially rescued the activity of ADP-ribosyl cyclase when both boric acid and methyl boric acid were added together as compared to boric acid alone (Fig. 4).

The Lineweaver and Burk plots of ADP-ribosyl cyclase inhibition by boric acid revealed extrapolated intercepts near the x-axis, implying that the inhibition of ADP-ribosyl cyclase by boric acid is mostly non-competitive (Fig. 5). Nonlinear regression of the curves fitted to the Michaelis–Menten equation showed that the K_m remained constant but that V_{max} changed, confirming that non-competitive inhibition occurred (Fig. 5, insert). The apparent K_i was determined to be 40.5 ± 0.5 mM (Eq. (2)).

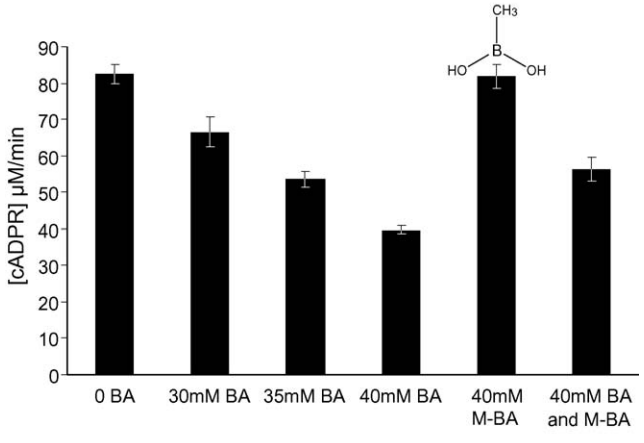


Fig. 4. Methyl boric acid (M-BA) does not inhibit ADP-ribosyl cyclase. The structure of methyl boric acid is shown in the figure. Boric acid (BA) inhibits the enzyme in a dose dependent manner (30–40 mM). The activity of the enzyme inhibited by BA is partially rescued by addition of M-BA ($n = 3$). $[NAD^+] = 2$ mM and reaction time = 0–2 min.

$$K'_i = \frac{[I]}{(V_{max}/V'_{max}) - 1} \quad (2)$$

3.3. ESI-MS analysis of borate complexed with cADPR

In WAT solvent (pH 10.3) the negative ion ESI-MS spectra of 100 μM solutions of cADPR revealed a prominent molecular anion for the singly charged, alkali metal-free molecule $[(M-H)^-]$ at m/z 539.8 (calc. 540.0 Da) (Fig. 6A). Following the addition of $^{11}B(OH)_3$ (500 μM final concentration), the spectra revealed additional signals at m/z 565.7, assigned as the singly charged boric acid-cADPR complex (calc. 566.0 Da for $(M-H)^-$), and m/z 583.7, assigned as a borate-cADPR complex (calc. 584.0 Da for $(M-H)^-$)(Fig. 6B). The sum areas of the m/z 539.8, 565.7, and 583.7 peaks correspond to the mass spectrometric response from the cADPR injected (1×10^{-9} mol). From the known concentrations of cADPR and boric acid, and the measured relative areas of free nucleotide and borate-complexed nucleotide, the concentration of the boric acid/borate-cADPR complex was calculated. Since the concentrations of all the different species in equilibrium (Eq. (3)) were known, the equilibrium constant was calculated (Eq. (4)). The calculated apparent K_A for borate-cADPR complex was 655 ± 99 L/mol.

$$K_{equ} = \frac{[B_{complex}]}{[B_{free}][Nuc_{free}]} \quad (3)$$

$$[Nuc_{total}] = [Nuc_{free}] + [Nuc_{adduct}] + [Nuc_{complexed}] \quad (4)$$

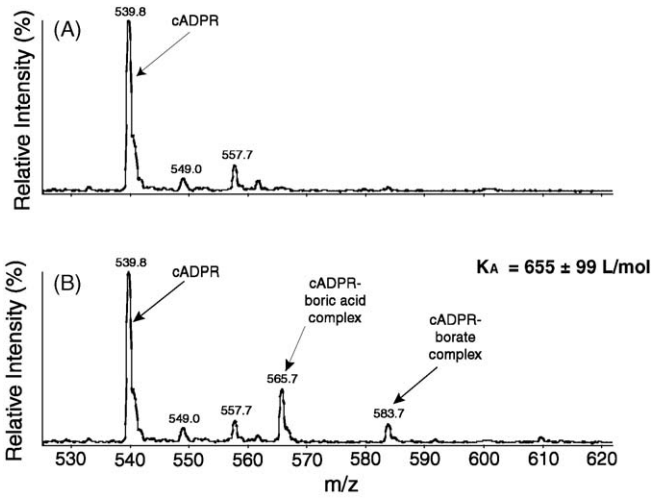


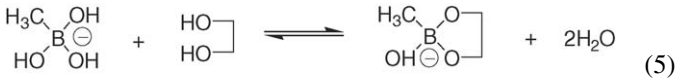
Fig. 6. Negative ion ESI-mass spectra of cADPR and cADPR–boric acid complexes in water:acetonitrile:triethylamine mixtures at pH 10.3. (A) 100 μM cADPR showing an intense $(M-H)^-$ signal at *m/z* 539.8 (calc. 540.0 Da). The signals at *m/z* 549.0 and 557.7 are assigned as unknown impurities in the cADPR sample. (B) A mixture of 100 μM cADPR and 500 μM $^{11}B(OH)_3$ produced signals corresponding to the cADPR–boric acid complex at *m/z* 585.7 (calc. 586.0 Da), and the cADPR–borate complex at *m/z* 583.7 (calc. 584.0). Apparent binding constant, K_A, for the complex formation was calculated to be 655 ± 99 L/mol.

4. Discussion

The source of the cyclase used in this study was *A. californica*, a herbivorous sea hare that lives in protected coves along the California coast. This creature normally inhabits the land-side of wave action in seaweed beds that protect it from predators and furnish a food supply. Fecal pellet analysis have determined that *A. californica* prefers to eat dark brown and green seaweeds pulverized by wave action [37]. Sea water on average contains 4.6 mg B/L [38], and seaweeds bioaccumulate boron, averaging about 94 mg B/kg, a value substantially higher than the boron concentration of more familiar leafy plants like lettuce which contain 1.0 mg B/kg.

In the present study, CE was used for the first time to determine and characterize the inhibition of ADP-ribosyl cyclase by boric acid. In the presence of boric acid, ADP-ribosyl cyclase was non-competitively inhibited, slowing the production of the Ca^{2+} mobilizing cADPR. The high concentrations of boric acid required to inhibit the cyclase in this study may reflect the sea hare's environmental adaptation and preference for consuming and living in a plume of fine sea weed particles. Furthermore, in previous work we showed by ESI-MS and ^{11}B NMR that the binding of boric acid to nucleotides is pH dependent and is significantly increased under more basic conditions [15]. If the pH near the binding site becomes more basic, a lower boric acid concentration would be required to inhibit the enzyme. Studies from CD38, a mammalian homolog of ADP-ribosyl cyclase, have suggested that cADPR production may be involved in regulating insulin secretion [39], muscarinic receptor-mediated Ca^{2+} signaling in pancreatic acinar cells [40], and in chemotaxis in response to the f-met-leu-phe peptide in neutrophils [41]. Furthermore, since cADPR is a Ca^{2+} mobilizing agent, it may even be involved in general biochemical processes such as muscle movement, neurotransmitter release, and cell proliferation [32]. The fact that the production of cADPR by *Aplysia* cyclase can be disrupted by boric acid suggest that the cyclase may be involved in boric acid anti-proliferation effect in human prostate cancer cell [42].

Methyl boric acid was used as a negative control for boric acid. The specificity of boric acid as an inhibitor of the cyclase was apparent as methyl boric failed to inhibit enzyme activity at equivalent concentrations. Furthermore, the use of methyl boric acid allowed controls for ionic strength and other unwanted changes due to the addition of boric acid. Boric acid-inhibited enzyme could be partially rescued by methyl boric acid, suggesting that the two compounds compete for the same binding site on the enzyme. Methyl boric acid has three hydroxyl groups in anionic form (Eq. (5)), and forms monoester bonds with single *cis*-diol groups, whereas boric acid can bind two *cis*-diol groups to form diester bonds (Eq. (1)). This difference is important in plants where a borate atom crosslinks polysaccharide chains through diester bonds formed between opposing apiose residues. This suggests that boric acid inhibition of ADP-ribosyl cyclase may require the formation of a diester bond near the active site.



(5)

Lineweaver and Burk plots were used to show that boric acid inhibited ADP-ribosyl cyclase non-competitively. Cornish-Bowden and Eisenthal plots were also used to confirm that the mode of inhibition was non-competitive (plot not shown) [43,44]. Boric acid has been shown to inhibit several oxidoreductase enzymes all of which use NAD^+ as a co-factor, including alcohol [45], lactate [46], phosphogluconate [47], glucose-6-phosphate [48], glyceraldehyde-3-phosphate [49], and succinate dehydrogenases [50]. The present work adds to this list ADP-ribosyl cyclase, an enzyme that uses NAD^+ as a substrate. The mammalian cyclase, CD38, is a multifunctional enzyme, with both cyclase and hydrolysis activity [6], whereas *Aplysia* ADP ribosyl-cyclase only has cyclase activity [10]. We used standard ADPR and determined its CE migration time to be 19.0 min (data not shown). The absence of a peak at this position in the electrophoretograms showed that the enzyme had no detectable hydrolase activity under the conditions of the assay. The inhibition of cADPR production by boric acid therefore cannot be attributed to alterations in the hydrolase activity of the enzyme.

Nicotinamide, a byproduct of the cyclase reaction, is a known inhibitor of ADP-ribosyl cyclase [51]. The linearity of the initial rate curves from 0 to 2 min showed that nicotinamide did not appreciably inhibit ADP-ribosyl cyclase as the concentration increased over time (data not shown). Upon addition of boric acid, initial rate curves remained linear, revealing that the presence of nicotinamide did not alter the inhibition of ADP-ribosyl cyclase by boric acid. We previously used ESI-MS/MS to observe fragments of the NAD^+-boric acid complex and determined that the binding sites were *cis*-diol groups on the riboses, and not the nicotinamide group [15]. The boric acid would there-

fore not be expected to interfere with the inhibition activity of nicotinamide on the cyclase. Therefore, these data support an interpretation that boric acid inhibited ADP-ribosyl cyclase directly.

In investigating the inhibitory effect of boric acid on the enzymatic activity of ADP-ribosyl cyclase, the K_m value was calculated to be 1.24 ± 0.05 mM. The apparent K_m derived in this study is greater than those reported by Migaud et al. (135 μM) [20] and Graeff et al. (39 μM) [52]. The reasons for this disparity are unclear. It is possible that the incubation temperature, buffer pH, and buffer concentration may have caused variation in the K_m value. Migaud et al. used 17 °C, Graeff et al. used ambient temperature (23–25 °C), and this study used 37 °C incubation temperatures. Since this enzyme is derived from a sea hare, it is possible that temperatures lower than the body temperature may increases the efficacy of the enzyme. In addition, Migaud et al. used 25 mM HEPES (pH 8) and Graeff et al. used 20 mM Tris (pH 7). In this study, 200 mM Tris (pH 7.4) was used because the high buffer concentration was necessary to control for the pH change after the addition of boric acid. Although there is a variation in the K_m value among these three studies, this study showed that boric acid inhibits ADP-ribosyl cyclase.

Boric acid binds to NAD^+ (1840 ± 110 L/mol) [15] as well as cADPR (655 ± 99 L/mol) (Fig. 6). In most cases, the best way to determine the apparent K_A of a complex formation is by titrating a ligand (i.e. boric acid) up to or through the expected dissociation concentration. In previous work, titration of boric acid to NAD^+ was used to determine that the apparent K_A was 1840 ± 110 L/mol [19], but the titration method could not be used for cADPR because of ion suppression at higher boric acid concentrations. We showed that the apparent K_A determination using a single concentration with $n = 12$ for NAD^+ complexation with boric acid gave a similar value as the titration method. Alkaline pH was employed to optimize the complexation of boric acid to cADPR. For comparison purposes with other nucleotides, the apparent K_A of cADPR with boric acid was also measured in alkaline pH.

The inhibition of ADP-ribosyl cyclase by boric acid is different from general inhibition because the boric acid–NAD^+ complex serves as an inhibitor. We have modified the general non-competitive inhibition model to construct a new model to fit the data (Fig. 7). Methyl boric acid did not inhibit the enzyme but rescued boric acid inhibition because of its competition with boric acid to form a complex with NAD^+ reduced the overall concentration of the boric acid–NAD^+ complex. The mechanism of the inhibition may be further complicated by the fact that boric acid binds cADPR. Since ADP-ribosyl cyclase does not have hydrolase activity the increase in cADPR is proportional to the increase in cADPR–boric acid complex. The increase in cADPR in the presence of boric acid did not cause deviation from the linearity at different reaction time points suggesting that cyclase inhibition was not associated with the formation of a cADPR–boric acid complex. Nevertheless, since cADPR interacts with the ryanodine receptor to release Ca^{2+}, cADPR–boric acid complex could affect its ability to trigger Ca^{2+} release from endoplasmic reticulum.

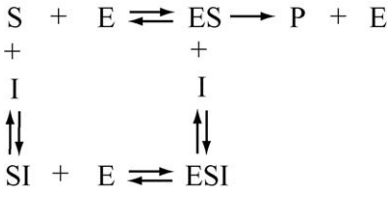


Fig. 7. Non-competitive inhibition model for ADP-ribosyl cyclase inhibition by boric acid. S = NAD^+, E = ADP-ribosyl cyclase, I = boric acid, and P = cADPR.

In order to determine if the high boric acid K_i is an adaptation of the *Aplysia* cyclase it will be necessary to evaluate CD38. The source of the enzyme was *A. californica*, a sea hare that feeds on seaweeds containing high concentrations of boron. Human cell-surface antigen CD38 is a 46 kDa type II glycoprotein with a single transmembrane domain [53]. In contrast to ADP-ribosyl cyclase, CD38 is an ectoenzyme with both NAD^+ glycohydrolase (NADase) and cADPR hydrolase activities that produce ADP-ribose from cADPR. Since CD38 is an ectoenzyme with catalytic domains facing away from the cytoplasm, it would encounter boric acid in the extracellular environment, but this would be expected to be in the micromolar range rather than the millimolar range experienced by *Aplysia*.

In conclusion, the present study determined that boric acid inhibits ADP-ribosyl cyclase non-competitively and binds directly to cADPR. CE proved to be an attractive alternative to HPLC for the analysis of cADPR. The results suggest that boric acid or NAD^+–boric acid complex inhibits ADP-ribosyl cyclase directly, possibly by forming a diester bond near the active site. To determine if the high boric acid K_i is the result of adaptation of *Aplysia* to a high boron environment, it will be necessary to study boric acid inhibition in the mammalian enzyme CD38, which is exposed to lower boron concentrations.

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References

[1] N. Rusinko, H.C. Lee, J. Biol. Chem. 264 (1989) 11725.
[2] H.C. Lee, R. Aarhus, Biochim. Biophys. Acta 1164 (1993) 68.
[3] W. Masuda, S. Takenaka, S. Tsuyama, H. Inui, K. Miyatake, Y. Nakano, J. Biochem. 125 (1999) 449.
[4] J. Sánchez, P. Duque, N.H. Chua, Plant J. 38 (2004) 381.
[5] H.C. Lee, Biol. Chem. 380 (1999) 785.
[6] H.C. Lee, R. Aarhus, Cell Regul. 2 (1991) 203.
[7] H. Higashida, M. Hashii, S. Yokoyama, N. Hoshi, X.L. Chen, A. Egorova, M. Noda, J.-S. Zhang, Pharmacol. Ther. 90 (2001) 283.
[8] H.C. Lee, Chem. Immunol. 75 (2000) 39.
[9] M. Howard, J.C. Grimaldi, J.F. Bazan, F.E. Lund, L. Santos-Argumendo, R.M. Parkhouse, T.F. Walseth, H.C. Lee, Science 262 (1993) 1056.
[10] E. Zocchi, L. Franco, L. Guida, U. Benatti, A. Bargellesi, F. Malavasi, H.C. Lee, A. De Flora, Biochem. Biophys. Res. Commun. 196 (1993) 1459.
[11] Y. Hirata, N. Kimura, K. Sato, Y. Ohsugi, S. Takasawa, H. Okamoto, J. Ishikawa, T. Kaisho, K. Ishihara, T. Hirano, FEBS Lett. 356 (1994) 244.

[12] M. Itho, K. Ishihara, H. Tomizawa, H. Tanaka, Y. Kobune, J. Ishikawa, T. Kaisho, T. Hirano, Biochem. Biophys. Res. Commun. 203 (1994) 1309.
[13] R. Aarhus, R.M. Graeff, D.M. Dickey, T.F. Walseth, H.C. Lee, J. Biol. Chem. 270 (1995) 30327.
[14] G.S. Prasad, D.E. McRee, E.A. Stura, Nat. Struct. Biol. 3 (1996) 957.
[15] D. Kim, B. Marbois, K. Faull, C. Eckhert, J. Mass Spectrom. 38 (2003) 630.
[16] R. Badugu, J.R. Lakowicz, C.D. Geddes, Bioorg. Med. Chem. 13 (2005) 113.
[17] W. Wang, X. Gao, B. Wang, Curr. Org. Chem. 6 (2002) 1285.
[18] N.D. Winblade, I.D. Nikolic, A.S. Hoffman, J.A. Hubbell, Biomacromolecules 1 (2000) 523.
[19] D. Kim, K. Faull, A. Norris, C. Eckhert, J. Mass Spectrom. 39 (2004) 743.
[20] M.E. Migaud, R.L. Pederick, V.C. Bailey, B.V. Potter, Biochemistry 38 (1999) 9105.
[21] P. Kießling, G.K. Scriba, F. Süß, G. Werner, H. Knoth, M. Hartmann, J. Pharm. Biomed. Anal. 36 (2004) 535.
[22] J.M. Fujima, N.D. Danielson, J. Capillary Electrophor. 7 (2002) 19.
[23] R.M. Graeff, T.F. Walseth, H.K. Hill, H.C. Lee, Biochemistry 35 (1996) 379.
[24] A.A. Suave, C. Munshi, H.C. Lee, V.L. Schramm, Biochemistry 37 (1998) 13239.
[25] K. Schweitzer, G.W. Mayr, A.H. Guse, Anal. Biochem. 299 (2001) 218.
[26] L. Sternfeld, E. Krause, A.H. Guse, I. Schulz, J. Biol. Chem. 278 (2003) 33629.
[27] D.M.F. Savarese, J.T. Russel, A. Fatatis, L.A. Liotta, J. Biol. Chem 267 (1992) 21928.
[28] A.R. Means, FEBS Lett. 347 (1994) 1.
[29] A.D. Short, J. Bian, T.K. Gosh, R.T. Waldron, S.L. Rybak, D.L. Gill, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 4986.
[30] E. Kohn, R. Allessandro, J. Spoonster, R. Wersto, L. Liotta, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 1307.
[31] P. Nicotera, B. Zhivotovsky, S. Orrenius, Cell Calcium 16 (1994) 279.
[32] M.J. Berridge, J. Physiol. 499 (1997) 291.
[33] R. Rowe, C. Eckhert, J. Exp. Biol. 202 (1999) 1649.
[34] P. Schmitt-Kopplin, N. Hertkorn, A.W. Garrison, D. Freitag, A. Kettrup, Anal. Chem. 70 (1998) 3798.
[35] S. Hoffstetter-Kuhn, A. Paulus, E. Gassmann, H. Widmer, Anal. Chem. 63 (1991) 1541.
[36] B.J. Glasgow, A.R. Abduragimov, T.N. Yusifov, O.K. Gasymov, J. Horwitz, W.L. Hubbell, K.F. Faull, Biochemistry 37 (1998) 2215.
[37] I. Kato, Y. Yamamoto, M. Fujimura, N. Noguchi, S. Takasawa, H. Okamoto, J. Biol. Chem. 274 (1999) 1869.
[38] Y. Fukushi, I. Kato, S. Takasawa, T. Sasaki, B.H. Ong, J. Biol. Chem. 276 (2001) 649.
[39] S. Partida-Sanchez, D.A. Cockayne, S. Monard, E.L. Jacobson, N. Oppenheimer, Nat. Med. 7 (2001) 1209.
[40] L.R. Winkler, The biology of California sea hares of the genus *Aplysia*, Ph.D. Dissertation, University of Southern California, Los Angeles, CA, 1957.
[41] R.W. Sprague, The Ecological Significance of Boron, U.S. Borax Research Corp., Anaheim, CA, 1972, p. 58.
[42] W.T. Barranco, C.D. Eckhert, Cancer Lett. 216 (2004) 21.
[43] A. Cornish-Bowden, R. Eisenthal, Biochem. J. 139 (1974) 721.
[44] A. Cornish-Bowden, R. Eisenthal, Biochim. Biophys. Acta 523 (1978) 268.
[45] A.H. Roush, B.B. Gowdy, Biochim. Biophys. Acta 52 (1961) 200.
[46] K.W. Smith, S.L. Johnson, Biochemistry 15 (1976) 560.
[47] S. Lee, S. Aronoff, Science 158 (1967) 798.
[48] M.Y. Shkol'nik, N.L. Il'inskaya, Fiziol. Rastenii. 22 (1975) 801.
[49] T. Misawa, H. Kaneshima, M. Akagi, Chem. Pharm. Bull. 14 (1966) 467.
[50] C.J. Shepherd, Biochem. J. (Tokyo) 48 (1951) 483.
[51] J.K. Sethi, R.M. Empson, A. Galione, Biochem. J. 319 (1996) 613.
[52] R. Graeff, T. Walseth, K. Fryxell, W. Branton, H.C. Lee, J. Biol. Chem. 269 (1994) 30260.
[53] D.G. Jackson, J.I. Bell, J. Immunol. 144 (1990) 2811.