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Sperm express a Ca^{2+} -regulated NAADP synthase

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Abbreviations Footnote: NAADP, nicotinic acid adenine dinucleotide phosphate; IP_3 , inositol 1,4,5-trisphosphate; cADPR, cyclic Adenosine Diphosphate Ribose; NACE, NAD(P)^+ catabolizing enzyme; NA, nicotinic acid.

Synopsis

NAADP (nicotinic acid adenine dinucleotide phosphate), the most potent Ca^{2+} -mobilizing second messenger, is active in a wide range of organisms and cell types. Until now, all NAADP producing enzymes have been thought to be members of the ADP-ribosyl cyclase family. ADP-ribosyl cyclases exhibit promiscuous substrate selectivity, synthesize a variety of products and are regulated in a limited manner, which may be non-physiological. We now report the presence of an enzyme on the surface of sea urchin sperm that exhibits bell-shaped regulation by Ca^{2+} over a range (EC_{50} of 10 nM and IC_{50} of 50 μM) that is physiologically relevant. Uniquely, this surface enzyme possesses complete selectivity for nucleotides with a 2'-phosphate and exhibits only base-exchange activity without any detectable cyclase activity. Taken together, these findings indicate that this novel enzyme should be considered as the first true NAADP synthase.

Introduction

Currently, there are four generally accepted Ca^{2+} -releasing second messengers: inositol 1,4,5-trisphosphate (IP_3) [1], cyclic ADP-ribose (cADPR) [2], sphingosine 1-phosphate [3] and nicotinic acid adenine dinucleotide phosphosphate (NAADP) [4]. Compared to IP_3 and cADPR, NAADP is unique in many respects including releasing Ca^{2+} from lysosome-related organelles [5], exhibiting potent self-desensitization [6, 7] which may relate to pseudo-irreversible binding [6], and the ability to initiate Ca^{2+} increases via either Ca^{2+} -induced Ca^{2+} release [8] or store overloading [9-11]. Nevertheless, many features of NAADP behaviour are contested or remain unknown. For example, the Ca^{2+} store targeted by NAADP has been suggested to be the endoplasmic reticulum [12] and the channel on which NAADP acts directly has been suggested to be the ryanodine receptor rather than a novel channel [13-15]. There is also a large gap in our understanding of the signalling steps involved in NAADP synthesis. Indeed, if NAADP is synthesized by base exchange, it is not entirely clear how this synthesis is regulated endogenously by enzymes categorized as ADP-ribosyl cyclases [16].

To date, all known ADP-ribosyl cyclases exhibit both cyclase (cyclic ADP-ribose synthesis) and base-exchange (NAADP synthesis) activity, with the relative ratio mainly regulated by pH and substrate availability [16] and moderately regulated by Zn^{2+} [17], phosphorylation and cyclic AMP and cyclic GMP [18, 19]. That is, ADP-ribosyl cyclases can perform a multitude of reactions dictated entirely by substrate availability (Figure 1): NAD is converted into cyclic ADP-ribose and nicotinic acid and NADP are converted into NAADP [20, 21]. One concern with all mechanisms of regulation described thus far is that the reaction conditions are not physiological relevant (e.g., pH of 4 or millimolar concentrations of Zn^{2+} and Cu^{2+}) [16, 20, 21].

Previously, we demonstrated that NAADP fulfilled all the criteria necessary to be a bona fide second messenger during sea urchin sperm activation [22]. We reasoned that given the capacity of sea urchin sperm to provide rapid and large synthesis of NAADP, sperm would be a good model system to study the regulation of NAADP synthesis. We now report that sea urchin sperm possess an enzyme capable of base exchange, with selectivity for nucleotides with a 2'-phosphate, that is regulated by Ca^{2+} in a bell-shaped manner. Moreover, within our limits of detection, this enzyme can only perform base exchange and not cyclization. Therefore, we propose that this enzyme is most appropriately termed NAADP synthase.

Experimental Procedures

Sperm collection

Sperm were shed from *Lytechinus pictus* sea urchins by intracoelemic injection of approximately 1 ml, 0.5 M KCl. Sperm were collected on a “dry” petridish and were transferred to a microcentrifuge tube and stored at 4°C until required.

Permeabilization and homogenization of sperm

Prior to permeabilization and homogenization, the viability of the sperm was tested visually under a microscope as determined by the induction of swimming upon dilution into artificial seawater, to ensure they were healthy. Sperm were permeabilized using 50 μ M digitonin. Homogenization was carried on using Jencons VC50 sonicator. 3 pulses of 50 watts, 50 Hz of 3 s were delivered to the samples, followed by freeze thawing (-80°C for 10 min and thawed at room temperature).

High performance liquid chromatography

Nucleotides were separated on a column (3 x 150 mm), packed with AGMP-1 resin (Bio-Rad Laboratories). Samples (200 μ l) were injected onto a column equilibrated with water. The bound material was eluted with a concave-up gradient of trifluoroacetic acid, which increased linearly to 2% at 1.5 min and to 4, 8, 16, 32 and 100% (150 mM trifluoroacetic acid) at 3, 4.5, 6.0, 7.5 and 7.51 min respectively. The flow rate was 4 ml/min. The nucleotides were detected by an absorbance at 254 nm.

ADP-ribosyl cyclase activity

The cyclase activity was determined by an assay that relies on the fluorescence increase caused by the formation of cGDP-ribose from NGD [23]. The reaction was carried out in the presence of 500 mM KCl, 4 mM NGD, 20 mM HEPES pH 7 and intact sperm (final concentration of 20% volume/volume). The fluorescence intensity was monitored using a Perkin Elmer fluorescence spectrometer at an excitation wavelength of 300 nm and an emission wavelength of 410 nm.

Ca²⁺ and Mg²⁺ buffer preparation

Solutions to contain varying concentrations of free Ca²⁺ were prepared using the pH-metric method described previously [24]. Absolute Ca²⁺ concentrations were later

verified with a Ca^{2+} -selective electrode. The computer program WinMax chelator (Version 2.05) was used to prepare Mg^{2+} solutions of different buffered concentrations [25].

Enzyme kinetic studies

Intact sperm were incubated (final concentration 20% volume/volume) with varying concentrations of NADP and 50 mM nicotinic acid at 23°C in the presence of 500 mM KCl and 20 mM HEPES for 1 h. Similarly, to find the K_m for nicotinic acid was varied in the presence of 5 mM NADP. The reaction was stopped after 1 h by addition of ice-cold ethanol and centrifugation at 16100 g. The protein pellet was stored at -20°C for protein analysis by bicinchoninic acid. The production of NAADP was determined using the high performance liquid chromatography method described above.

Materials

Lytechinus pictus sea urchins were obtained from Marinus Inc. (Long Beach, California, USA). All chemicals were from Sigma except where indicated.

Analysis

Data are presented as mean \pm standard error of the mean. Statistical comparisons were performed by a non-paired t-test. Enzyme kinetic analysis was performed with and presented using GraphPad Prism.

Results and discussion

We explored NAADP synthesis via base exchange (Figure 1) in sea urchin sperm by adding exogenous NADP and nicotinic acid to sperm permeabilized with digitonin to facilitate the entry of substrates (Figure 2A,B). Permeabilized sperm performed the base-exchange reaction (Figure 2B). Surprisingly, the non-permeabilized sperm, which was our negative control, synthesized NAADP in comparable levels to the permeabilized sperm (Figure 2C), suggesting the presence of an enzyme on the surface of the sperm. We verified that sperm were permeabilized by digitonin with trypan blue uptake (Figure 2D). Also, there was no additional enzymatic activity in freeze-thawed and sonicated sperm (Figure 1E), providing further evidence that all the enzymatic activity was on the surface. To localize the enzymatic activity on the sperm, we decapitated sperm with shear (passing through a 25-gauge needle for 7 times) and then separated the heads and tails via density gradient centrifugation [26, 27]. We verified that the resulting fractions were highly enriched in heads or tails by visible light microscopy (Figure 2F). NAADP production per mg protein was about four times higher in the isolated tails than the isolated heads (Figure 2G). Taken together, these data demonstrate that sperm contain a base-exchange activity on their surface.

Having found an enzyme on the surface of the sperm that is capable of producing NAADP (Figure 2), we investigated if this enzyme was functionally similar to any of the cyclases already characterized in regard to substrate selectivity, enzyme kinetics and regulation (Table 1) [20, 21, 28]. All of the NAADP synthesising ADP-ribosyl cyclases reported so far that are capable of producing NAADP, are multifunctional in nature, and exhibit four activities: base exchange, glycohydrolase, hydrolase and cyclase (Figure 1) [20, 21]. All these reactions can be explained by a unifying enzymatic mechanism involving a covalent intermediate [20, 29, 30]. Variations in the relative amounts of each of these enzymatic activities are characteristic of different ADP-ribosyl cyclase family members (Table 1). The extremes of the variations are illustrated by the contrast between *Aplysia* cyclase, which is entirely a cyclase, and CD38, which is mostly a hydrolase [31].

We examined both substrate selectivity and the range of many enzymatic activities possible with cyclases, specific to our enzyme present on the surface of sperm. Sperm readily produced NAADP by base exchange (Figure 3G; 1.72 ± 0.42 pmol/mg/min) but failed to produce detectable cyclic ADP-ribose phosphate (Figure 3A) or cyclic ADP-ribose (Figure 3B). A predominance of base exchange over cyclase

activity is unusual and limited to NACE, a cyclase present in *S. masoni* [32]. NACE itself does cyclize the NAD analogue NGD to cyclic GDP-ribose, which is hydrolysis resistant and fluorescent (300 nm excitation and 410 nm emission) [23]. In contrast, incubation of sperm with NGD did not produce detectable cyclic GDP-ribose (Figure 3H), whereas spiking the same sample with authentic ADP-ribosyl cyclase from *Aplysia* showed production of cyclic GDP-ribose. The inability of the sea urchin enzyme to use NGD as a substrate proves that it is distinct from NACE. That NGD is not a substrate, also suggests that the enzyme activity we have observed is not that of the recently characterized sea urchin ADP-ribosyl cyclase isoform C1 (Sparc C1), which was recently characterized [33]. Sperm exhibited pronounced glycohydrolase activity when incubated with NADP (Figure 3A). However, this activity was minimal when incubated with NAD (Figure 3B; 1.2%). In regard to hydrolase activity, the sperm surface enzyme did not convert cyclic ADP-ribose to ADPR (Figure 3C), cADPR-P to ADPR-P (Figure 3D) or NAADP to ADPR-P (Figure 3E). That the sperm enzyme could convert a small amount of NAD to ADPR but could not produce any cADPR (Figure 3B), or hydrolyze cADPR to ADPR (Figure 3C), indicates that the surface enzyme does not exhibit any cyclase activity. These results also demonstrate that both the base-exchange and glycohydrolase reactions exhibit selectivity for NADP, likely reflecting a requirement for both nicotinamide base and a 2'-phosphate. Taken together, these data demonstrate that the sperm surface enzyme is unlike any previously characterized ADP-ribosyl cyclases (Table 1) [32]. It is important to note that classical NADase enzymes, such as the NADase from *Neurospora*, do not catalyze base exchange (Figure 3F). Similarly, CD38 only hydrolyzes NADP, it does not cyclize it to cADPR-P [16, 34]. As sperm did not show detectable cyclase activity (Figure 3A,B), enzymatically, this enzyme is not a cyclase; therefore, we suggest that it is more correctly termed an NAADP synthase.

We next established the K_m and the V_{max} of NAADP synthase. Plots of initial velocity versus NADP concentration (with 100 mM nicotinic acid) and initial velocity versus nicotinic acid concentration (with 10 mM NADP) revealed a rectangular hyperbola that fit the Michaelis-Menten equation by non-linear regression (Figure 4A,B). Such a plot is consistent with a single binding site and no cooperativity. For ease of presentation of these data, double reciprocal plots are also given for nicotinic acid (Figure 4C) and NADP (Figure 4D). For nicotinic acid the K_m was 2.8 mM and the V_{max} was 5.3 pmol/mg protein/min (Figure 4A), whereas for NADP, the K_m was 111 μ M and the V_{max}

was 5.6 pmol/mg protein/min (Figure 4B). These values are different from all other characterized ADP-ribosyl cyclases that exhibit K_m values in the range of 200 μ M for NADP and 10 mM for nicotinic acid [19] (Table 1). Note that our enzyme kinetics measurements were not confounded by degradation of NAADP, as the sperm did not significantly metabolize NAADP over the time course of the assays (Figure 3E). Overall, the enzyme kinetic parameters provide additional support for the novelty of this enzyme.

ADP-ribosyl cyclase activity is highly sensitive to pH, with acidic pH favouring base exchange due to the protonation state of a critical aspartate at the active site [35]. The base-exchange activity of the sperm surface enzyme was optimal at pH 5 (Figure 5A), as is the case with other members of the cyclase family [16] (Table 1). Other ADP-ribosyl cyclases, such as CD38, have almost no base-exchange activity at pH 7 and can only catalyze the hydrolysis of NADP to ADPR-P [16]. The only exception is Aplysia cyclase, which shows a small amount of base-exchange activity [16]. Surprisingly, at pH 7 the sperm surface enzyme was capable of producing NAADP by base exchange at 25 percent of its pH optimum (1.8 ± 0.23 pmol/mg/min at pH 7 versus 7.1 ± 0.34 pmol/mg/min at pH 5). In regard to pH regulation, with the exception of Aplysia cyclase, the sperm base-exchange enzyme differs from all other members of the ADP-ribosyl cyclase family.

Previously, it has been shown that ADP-ribosyl cyclase enzymes are modulated by cations (Table 1). We studied the possible effect of Ca^{2+} on the base-exchange enzyme. Sperm were incubated in solutions containing different amounts free Ca^{2+} buffered with EGTA [24]. The response to Ca^{2+} concentration was bell-shaped: as the concentration of Ca^{2+} was increased, NAADP production was stimulated with an EC_{50} of 10 nM (Figure 5B) and then inhibited with an IC_{50} of 50 μ M (Figure 5B). This regulation is selective for Ca^{2+} because Mg^{2+} did not alter NAADP production (Figure 5C). Regulation by Ca^{2+} is unique to the sperm surface enzyme as all other ADP-ribosyl cyclases have been shown to be insensitive to Ca^{2+} [31, 36, 37](Table 1). Remarkably, the Ca^{2+} stimulation occurred at pH 7 and elevated the V_{max} to about half the velocity (Figure 5B) obtained by lowering the pH to 5 (Figure 3C). Taken together, the data suggest that either Ca^{2+} or pH can serve as an endogenous regulator of NAADP synthesis.

Other Ca^{2+} releasing pathways have several steps that respond to positive feedback by Ca^{2+} ; the NAADP-mediated pathway also appears to have positive feedback, but at different stages of signalling cascade. For example, both ryanodine receptors and

IP₃ receptors have feedback at the level of the channel enabling Ca²⁺-induced Ca²⁺ release [38], and the IP₃ pathway is stimulated by Ca²⁺ acting on phospholipase Cdelta-1 [39]. As NAADP-mediated Ca²⁺ release is insensitive to Ca²⁺ [40], Ca²⁺ feedback appears to occur exclusively at the enzymatic steps for synthesis (Figure 4) and metabolism [41], which may underlie NAADP-mediated Ca²⁺ oscillations [11, 41].

Considering that many of the other enzymes responsible for producing NAADP and cADPR are surface bound or in the lumen of organelles [33, 42, 43], the location of this enzyme may not be a great surprise. Indeed, a recent report of the molecular identity of the urchin ADP-ribosyl cyclases suggests that the enzyme is functional within the lumen of the endoplasmic reticulum [33]. Combining the conclusions from the study by Churamani et al. [33] with our current results leads us to propose a mechanism for the regulation of NAADP synthase by Ca²⁺ and pH over concentration ranges that are physiologically relevant when put into the context of the events occurring during fertilization (Figure 6). At fertilization the sperm and egg fuse and their plasma membranes become contiguous [44]. Subsequently, the sperm contents are engulfed by the egg and the plasma membrane of the sperm is endocytosed (Figure 6) [44]. It would be particularly informative to evaluate the possible locations of the NAADP synthase within the fertilized egg in regard to the physiological pH and Ca²⁺ concentrations. In terms of regulatory control, the most effective location for NAADP synthase would be in the lumen of vesicles (Figure 6). A luminal location is consistent with location of the sea urchin egg ADP-ribosyl cyclase 1C, which is present and active in the lumen of the endoplasmic reticulum when expressed in *Xenopus* eggs [33].

If NAADP synthase is widely distributed in many tissues and species, the results from this study will have far-reaching importance, as this synthase may be the physiologically relevant enzyme responsible for agonist-induced NAADP increases. Currently, although all ADP-ribosyl cyclases can make NAADP by base exchange, there is no direct evidence that this is the physiologically relevant pathway [45, 46]. Such base-exchange activity may be nothing more than a consequence of a covalent intermediate production in the cyclization reaction mechanism [20, 29]. Indeed, based on experiments with CD38 knockout mice, agonist-induced NAADP increases are unaffected, demonstrating that CD38 is of questionable importance for NAADP synthesis [45, 46].

In summary, we have uncovered an enzymatic activity, that cannot only synthesize NAADP by base exchange, but also is unique in its regulation by Ca²⁺ and

selectivity for NADP. Unlike all previous enzymes that can synthesize NAADP, which are all members of the family of enzymes termed ADP-ribosyl cyclases, this new enzyme is regulated by physiologically relevant factors and shows no detectable cyclase activity making it more appropriately termed an NAADP synthase.

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Stage 2(a) POST-PRINT

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Figure Legends

Figure 1. Enzymes in the ADP-ribosyl cyclase family are multifunctional and catalyze four classes of reaction: base exchange, cyclase, glycohydrolase and hydrolase. Although several exogenous nucleotides and pyridine bases can serve as substrates, NADP is used to illustrate each reaction.

Figure 2. Sperm exhibit base-exchange activity on their surface. (A) High performance liquid chromatography trace of the substrates (NADP and nicotinic acid) before incubation with sperm. Note that ADPR-phosphate (ADPR-P) is an impurity in commercial NADP. (B) Permeabilized sperm synthesize NAADP by base exchange. High performance liquid chromatography trace taken one hour after incubation of digitonin (50 μ M)-permeabilized sperm incubated with NADP (1 mM) and nicotinic acid (10 mM) in artificial seawater (20 mM HEPES, pH 7.0). (C) Intact sperm synthesize NAADP by base exchange. Reaction conditions were as in B except without digitonin. (D) Effect of digitonin concentration on sperm permeabilization. Permeabilization was assessed by incubating sperm with Trypan Blue for 5 min and then scoring the number of blue sperm heads in a sample of 100 viewed with bright-field microscopy. (E) Intact sperm shows comparable amounts of base-exchange activity to digitonin-permeabilized and freeze-thawed sperm. Data are mean \pm standard error of the mean, n=3. (F) Sperm heads and tails can be separated and purified. Visible light images of bands formed in a sucrose gradient after centrifugation of sperm beheaded by the shear force when passed through a needle. (G) Both sperm heads and tails exhibit base-exchange activity. Note that the headpiece of the sperm remains with the tail with this procedure. Data are mean \pm standard error of the mean, n=3.

Figure 3. The base-exchange enzyme on the surface of sperm shows unique substrate selectivity. All traces show high performance liquid chromatography separation of substrates and products (as labelled). (A, B) Determination of cyclase and glycohydrolase activity using the endogenous substrates NADP and NAD. Inset shows a magnified view of the baseline to demonstrate the absence of cADPR,

which elutes between NAD and ADPR. **(C, D, E)** Determination of hydrolase activity assessed with cADPR, cADPR-P and NAADP. The sharp peak preceding and distorting the cADPR peak is due to a small presence of NAD. **(F, G)** Determination of base-exchange activity of *Neruospora* NADase and sea urchin sperm. **(H)** Determination of cyclase activity of sperm and *Aplysia* ADP-ribosyl cyclase using NGD. The product of NGD cyclization is cyclic GDPR, which is fluorescent and hydrolysis resistant. The trace shows a sample incubated in a fluorimeter with excitation of 300 nm and emission of 410 nm, with slit widths of 5 nm. Sperm was added at 10 percent (volume/volume). *Aplysia* ADP-ribosyl cyclase was added at 100 $\mu\text{g/mL}$.

Figure 4. Base-exchange activity of the sperm surface enzyme shows Michaelis-Menten kinetics. **(A, B)** Linear-linear plots of substrate concentration versus initial velocity. **(C, D)** Double-reciprocal (Lineweaver-Burke) plots of substrate concentration and initial velocity.

Figure 5. Synthesis of NAADP by base exchange is regulated by pH and Ca^{2+} but not Mg^{2+} . **(A)** Effect of pH on the initial velocity of the base-exchange reaction. Results shown are mean \pm standard error of the mean, $n=3$. **(A, B)** Effect of Ca^{2+} or Mg^{2+} on the initial velocity of NAADP synthesis. All the reactions contained 1 mM NADP, 10 mM nicotinic acid, pH 7, 21°C and were started with the addition of $\sim 20 \mu\text{L}$ sperm ($\sim 1 \text{ mg protein/mL}$). Results shown are mean \pm standard error of the mean, $n=3-4$.

Figure 6. Possible physiological significance of a Ca^{2+} -regulated NAADP synthase. The two inset graphs show the dose-response curves for modulation of the NAADP synthase by Ca^{2+} and pH. The horizontal bars show the environmental and physiological concentrations of Ca^{2+} and protons. The cartoon highlights sperm-egg fusion and the possible subsequent events that would modulate NAADP synthase activity. 1. NAADP synthase enzyme (E) on the surface of the sperm would be inactive in sea water. 2. Sperm-egg fusion and mixing of their plasma membranes would not activate the enzyme. 3. Endocytosis of the NAADP synthase enzyme would make it luminal and expose it to Ca^{2+} concentrations in

the range capable of regulating its activity. 5. Loss of endosome membrane integrity would expose the enzyme to cytosolic Ca^{2+} fluctuations. Taken together, it is most reasonable that the NAADP synthase would be found in the lumen, as this would enable regulation.

Stage 2(a) POST-PRINT

Table 1. Comparison of enzyme kinetics, regulation and enzymatic activities of known ADP-ribosyl cyclases with sperm surface NAADP synthase

Abbreviations: nr, not reported and na, not applicable.

Parameter	Enzyme or Enzymatic Activity											
	CD38 (external) [16, 47]	CD157 (external) [37]	Aplysia cyclase (luminal) [16]	Human sperm [17] [48]	Brain cyclase [49]	Vascular smooth muscle [50]	NACE, <i>Schistosoma mansoni</i> [32]	Urchin Egg soluble (internal) [18, 19]	Urchin egg membrane bound (internal) [18, 19]	Urchin Soluble (ER lumen) [33]	Urchin Sperm	Urchin Sperm surface NAADP synthase (external)
Zn	Activates [51] Inhibits hydrolase, [52]	Activates [37]	Activates [50]	Switches cyclase/glycylolase activity [17]	Inhibits [49]	Inhibits [50]	nr	nr	Increase, [50]	nr	nr	nr
Cu	Activates [50, 51]	Inhibits mM [37]	nr	No effect [17]	Complete inhibition, 10 µM [49]	Inhibits [50]	nr	nr	nr	nr	nr	nr
Ca	No effect [51]	No effect	No effect	No effect [17] [48]	No effect [49]	nr	nr	nr	nr	nr	nr	Low-activates; High inactivates
Km NADP	1 mM [53]	nr	nr	nr	nr	nr	14 µM [32]	200 µM [18, 19]	0.2 mM [19] 0.4 mM [18]	nr	nr	110 µM
Km NA	5 mM [16]	nr	~10 mM [54]	nr	nr	nr	nr	10 mM [18, 19]	10 mM [19] 9 mM [18]	nr	nr	4 mM
Km NAD	15 µM [23, 29] 200 µM [17]	nr	39 µM [54]	150 µM [17] 8 µM [48]	21 µM [49]	37 µM [50]	39 µM [32]	660 µM (-cGMP) [19] 500 µM (+cGMP) [19]	nr	nr	90 µM [27]	Not a substrate
pH, cyclase	6-8 [16]	4-6.5 [37]	6-9 [54]	6.8 [17] 7 [48]	6-7 [49]	nr	na	7-8, [19]	7-8 [18, 19]	nr	nr	na
pH, base-exchange	4 optimal >7, nil [6, 29]	nr	4-5 optimal >7, active [54]	nr	nr	nr	nr	5 optimal >7 active [19]	5 optimal >7, active [18, 19]	4 active [33]	nr	5 optimal >7, active
Km NGD	1 µM [23] 2 µM [29]	nr	2.3 µM [23]	nr	Not a substrate [49]	nr	23 µM [32]	nr	nr	nr	nr	Not a substrate
cAMP	nr	nr	nr	nr	nr	nr	nr	No effect, 2	No effect [19] Activates base exchange [18]	nr	nr	nr
cGMP	nr	nr	nr	nr	nr	nr	nr	Activates cyclase [18, 19] No effect on base exchange [18, 19]	No effect [18]	nr	nr	nr
Nitric Oxide	S-nitrosylation [55]	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr
Cyclase												
NAD to cADPR	Yes [16]	Yes [37]	Yes [16]	Yes [17] [48]	Yes [49]	Yes [50]	Minimal [32]	Yes [18, 19]	Yes [18, 19]	Yes [33]	Yes [27]	No
NADP to cADPR-P	No [16, 34]	nr	Yes [16]	No [17]	nr	nr	nr	nr	nr	nr	nr	No
NGD to cGDPR	Yes [23]	nr	Yes [23]	No [17]	No [49]	Yes [50]	Yes [32]	Yes [18, 19]	Yes [18, 19]	Yes [33]	nr	No
Glycylolase												
NAD to ADPR	Yes [16]	nr	Yes [16]	Yes [17]	Yes [49]	nr	Yes [32]	nr	nr	nr	Yes [27]	Minimal
NADP to ADPR-P	Yes [16, 34]	nr	Yes [16]	nr	nr	nr	Yes [32]	nr	nr	nr	nr	Yes
NGD to GDPR	Weak [23]	nr	No [23]	nr	nr	Yes [50]	Yes [32]	nr	nr	nr	nr	No
Base exchange												
NADP + NA to NAADP	Yes [16]	nr	Yes [16]	nr	nr	nr	Yes [32]	Yes [18, 19]	Yes [18, 19]	Yes [33]	nr	Yes
Hydrolase												
cADPR to ADPR	Yes [16] Yes [34]	Yes [37]	No [23]	Yes [17] [48]	nr	nr	nr	Yes [18, 19]	Yes [18, 19]	nr	Yes [27]	No
cADPR-P to ADPR-P	No [16] No [34]	nr	nr	nr	nr	nr	nr	nr	nr	nr	nr	No
cGDPR to GDPR	No [23]	nr	No [23]	nr	nr	Yes [50]	nr	nr	nr	nr	nr	No

Figure 1

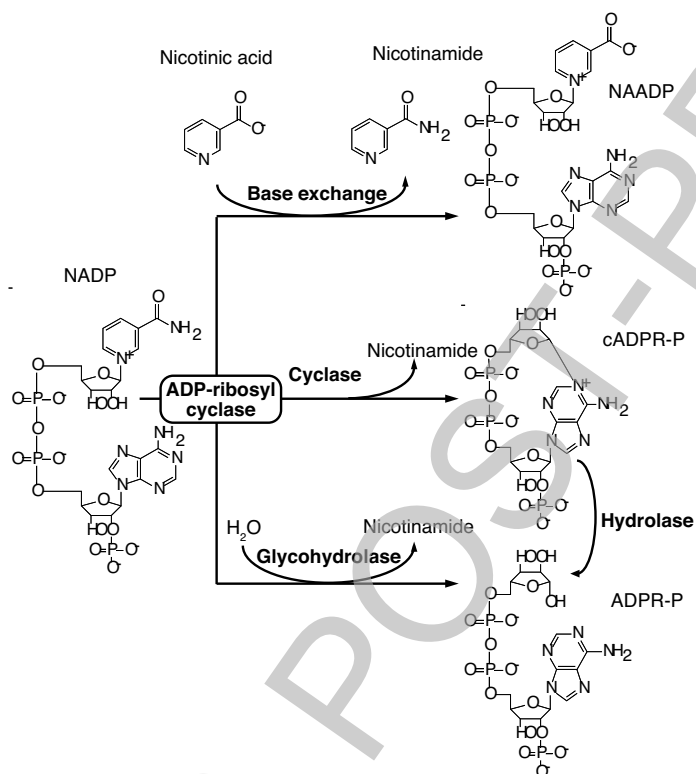


Figure 2

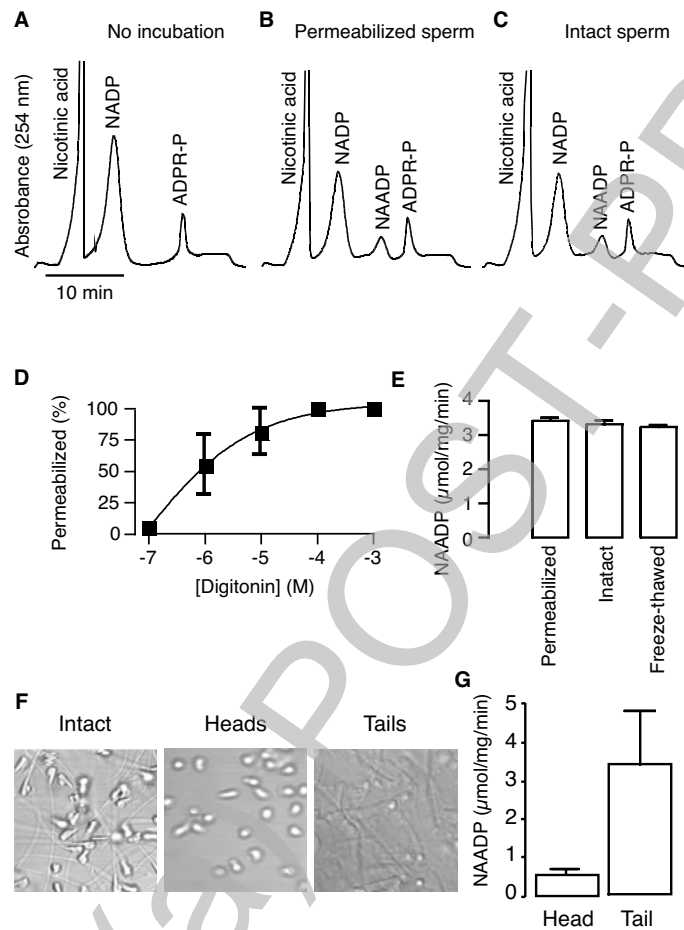


Figure 3

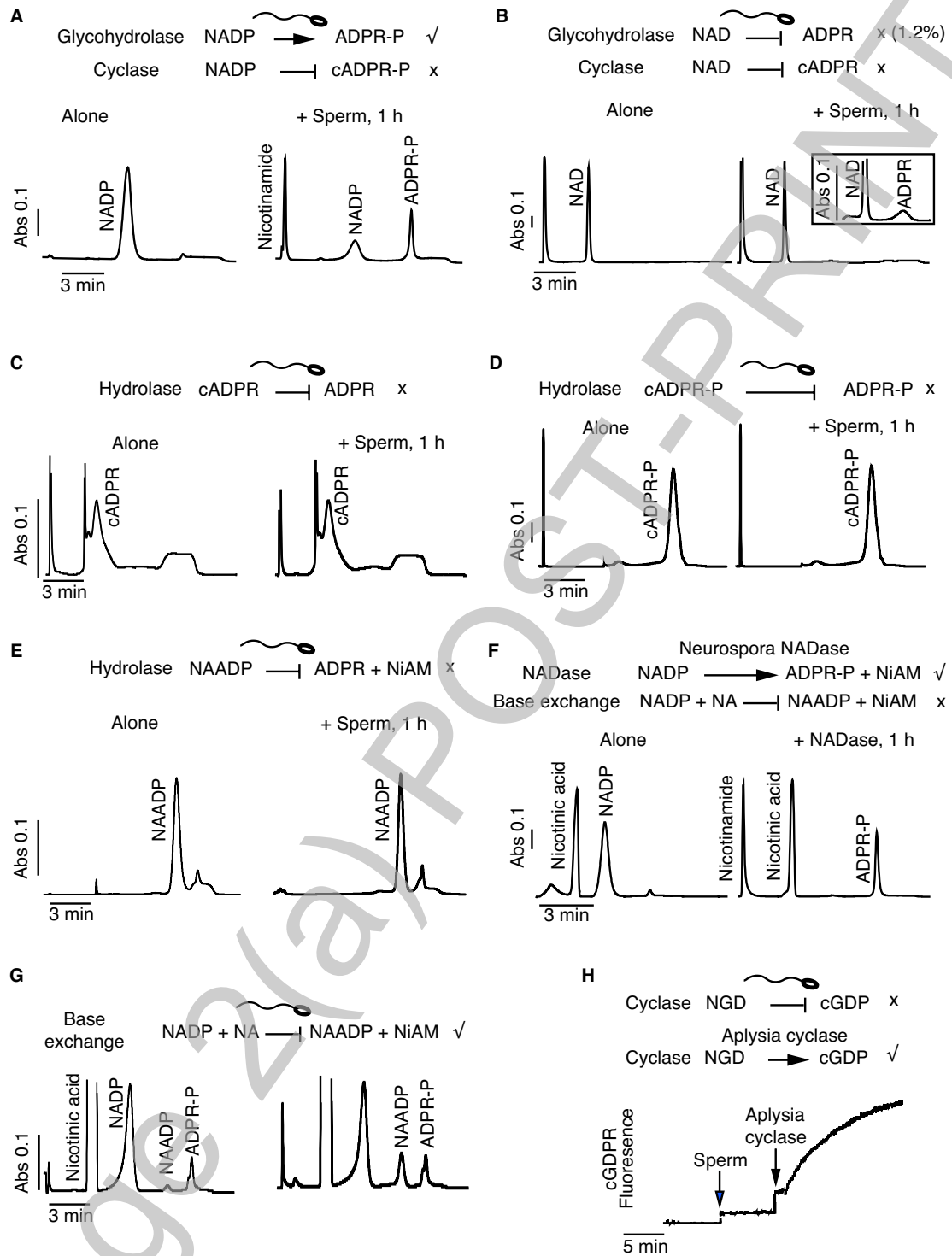


Figure 4

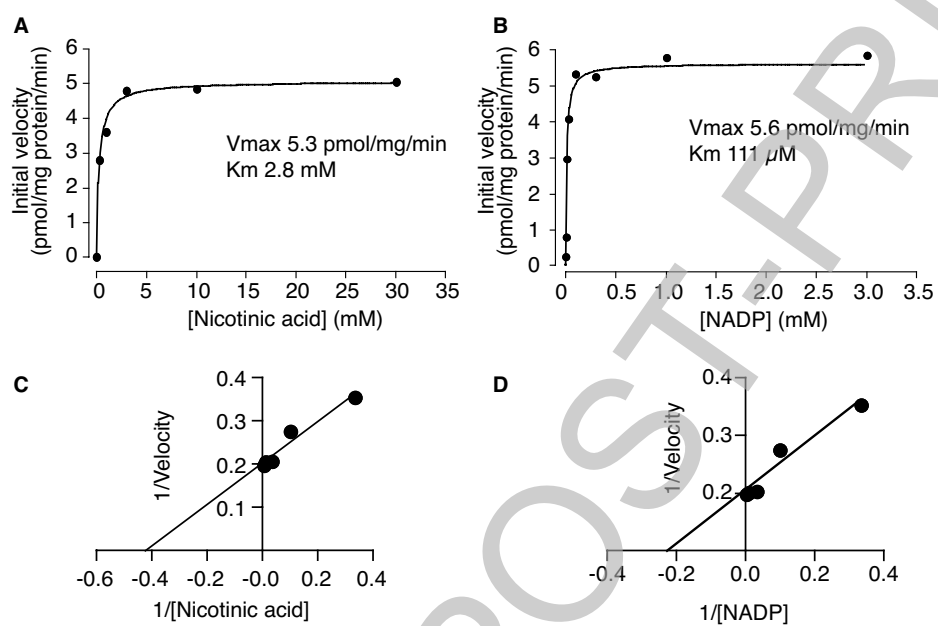


Figure 5

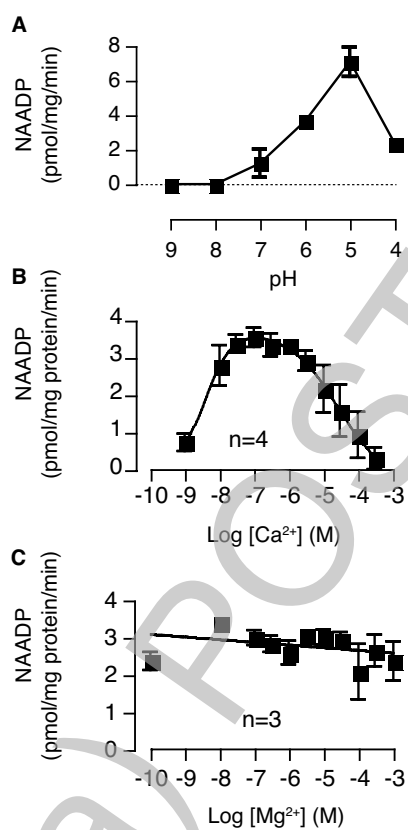


Figure 6

