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ORIGINAL ARTICLE

Thio-NADP Is Not an Antagonist of NAADP

Deborah M. Dickey,[1] Robert Aarhus,[2] Timothy F. Walseth,[1] and Hon Cheung Lee*,[2]

Departments of [1]Pharmacology and [2]Physiology, University of Minnesota, Minneapolis, MN 55455

ABSTRACT

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a metabolite of NADP, which can release Ca^{2+} from stores that are distinct from those activated by either cyclic ADP-ribose or inositol 1,4,5-trisphosphate (IP_3). It has previously been suggested that thio-NADP is a specific antagonist of NAADP (Chini et al. [1995] *J. Biol. Chem.* **270,** 3216–3223). Its effects in sea-urchin egg homogenates were investigated. At 50 μ*M*, thio-NADP activates partial Ca^{2+} release and totally inhibits subsequent challenge with a saturating concentration of NAADP. Purification by HPLC eliminates the Ca^{2+} releasing activity of 50 μ*M* thio-NADP and reduces the subsequent inhibition by 73.7 ± 1.3%. The residual inhibitory effect is no more than that exerted by 50 μ*M* of either NADP itself or nicotinic acid adenine dinucleotide (NAAD). These results are confirmed by ^{32}P-NAADP binding studies. Unpurified thio-NADP inhibits the specific ^{32}P-NAADP binding to egg microsomes with an IC_{50} of 40 μ*M*. After HPLC purification, only 20% inhibition is seen at a concentration as high as 50 μ*M*, similar to the extent of inhibition effected by 40 μ*M*

*Author to whom all correspondence and reprint requests should be addressed. E-mail: leehc@maroon.tc.umn.edu. Web site: http://enlil.med.umn.edu/www/phsl/faculty/hcl1.htm

NADP. These results indicate the inhibitory substance in thio-NADP is a contaminant. The partial Ca^{2+} release activity of unpurified thio-NADP suggests the contaminant is NAADP itself. This is supported by the fact that pretreatment with a sub-threshold concentration of only 2 n*M* NAADP totally desensitizes the egg homogenates such that no Ca^{2+} response is seen with saturating NAADP. Estimation from the binding studies shows that a contamination of 0.012% of NAADP in the unpurified thio-NADP samples is sufficient to account for the inhibitory effects. These results indicate thio-NADP is not an antagonist of NAADP.

Index Entries: NAADP; Ca^{2+} release; Thio-NADP.

INTRODUCTION

Three mechanisms for mobilizing intracellular Ca^{2+} stores are present in sea-urchin eggs. They are independently regulated by three different Ca^{2+} release activators, inositol 1,4,5-trisphosphate (IP_3), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP). The characteristics of the IP_3-sensitive Ca^{2+} release are similar to those described in many other systems and can be blocked by heparin, an antagonist of the IP_3-receptor *(1)*. Increasing evidence indicates cADPR is a modulator of the Ca^{2+}-induced Ca^{2+} release mechanism *(2–5)*, which is believed to be mediated by the ryanodine receptors. In contrast to IP_3 and cADPR, NAADP activates an hitherto unknown release mechanism. It is not affected by heparin or 8-amino-cADPR, selective inhibitors of the IP_3- and cADPR-induced Ca^{2+} release, respectively *(6)*. L-type Ca^{2+}-channel blockers selectively inhibit the NAADP-induced Ca^{2+} release, but have no effect on either the IP_3 or cADPR systems *(7)*. Fractionation studies show that the Ca^{2+} stores sensitive to NAADP can be separated from those sensitive to IP_3 and cADPR *(6)*. Thapsigargin, an inhibitor of the Ca^{2+} pump in the endoplasmic reticulum, selectively discharges the IP_3- and cADPR-sensitive Ca^{2+} stores, but not those sensitive to NAADP *(8)*. These results indicate that NAADP not only activates a totally independent Ca^{2+} release pathway, but the stores it acts on are also different from those sensitive to either IP_3 or cADPR. Another novel and distinguishing property of the NAADP system is that nonstimulatory concentrations of

NAADP, in the nanomolar range, can self-inactivate the release mechanism in a time- and concentration-dependent manner *(7,9)*. Ligand binding studies show that this self-inactivation process occurs at the level of the receptor *(7,9)*.

A selective antagonist for the NAADP system would be a useful tool for studying the mechanism by which NAADP induces Ca^{2+} release and also for examining the function of NAADP in biological systems. Chini et al. reported that thionicotinamide-NADP (thio-NADP) is a selective antagonist of NADP *(10)*. However, we have observed variable results with thio-NADP that appeared to correlate with the purity of the compound. In this study, we demonstrate that the inhibitory effects of thio-NADP are largely due to a contaminant in commercial thio-NADP preparations that is most likely NAADP itself. Results show that contamination of the unpurified thio-NADP with as little as 0.012% of NAADP, acting through the self-inactivation process, is sufficient to account for the inhibitory effects observed.

MATERIALS AND METHODS

Ca^{2+}-Release Assays

Homogenates (1.25%) of *Strongylocentrotus purpuratus* eggs were prepared as described previously *(9)*. Briefly, 25% homogenates were diluted with an intracellular medium (IM) consisting of 250 m*M* potassium gluconate buffer (pH 7.2), 0.5 m*M* ATP, 4 m*M* creatine phosphate, 2 U/mL creatine kinase, and 3 μ*M* fluo-3. The dilutions and all experiments were conducted at 17°C. Free Ca^{2+} concentrations were measured by monitoring the fluorescence (490 nm excitation, 535 nm emission) of the indicator, fluo-3. The experiments were performed with 0.2 mL of homogenate in a cuvet with the contents continuously mixed with a magnetic stir bar. Samples were added in 1- to 5-μL aliquots.

[^{32}P]NAADP Binding Assay

Microsomes were purified from 25% egg homogenates by Percoll density gradient centrifugation as described previously *(6)*. Briefly, 2 mL of the 25% egg homogenate were layered on 9 mL of 25% Percoll made up in the IM medium and separated by centrifugation. Three milliliters, containing the two distinct membrane bands, were collected from the middle of the gradient. Fractions

were aliquoted and stored at –20°C until use. The binding reactions were also performed as previously described *(9)*. In brief, the reactions were initiated with the addition of microsomes to tubes containing [^{32}P]NAADP and competitors. After 30 min incubation on ice, the reactions were filtered onto GF/C glass filters that had been presoaked in 10% PEG in the IM medium. The microsomes retained by the filters were washed twice with 3 mL of cold 10% PEG (in the IM medium). Binding of [^{32}P]NAADP was detected by liquid scintillation.

HPLC Purification of thio-NADP

Stock solutions (800 µL of 1 m*M*) of commercial thio-NADP, NADP, nicotinic acid adenine dinucleotide (NAAD), and NAADP were purified over a Mono-Q HR 5/5 column using a 1-*M* triethylammonium bicarbonate, pH 8.8 (TEAB) gradient or an AG MP-1 (0.3 × 15 cm) column using a 150-m*M* trifluoroacetic acid (TFA) gradient, as indicated. In both cases, the flow rate was 1 mL/min. The gradient in both cases was the same starting at 1% B (solvent B is 1 *M* TEAB, pH 8.8, for MONO Q or 150 m*M* TFA in water for AG MP-1, and solvent A is water for both systems). The gradient was held at 1% B for 1 min, increased linearly to 4% from 1 to 7.5 min, increased linearly to 16% B from 7.5 to 14.5 min, increased to 32% B from 14.5 to 21.5 min, increased linearly to 64% B from 21.5 to 28.3 min, stepped to 100% B from 28.3 to 30 min, held at 100% B from 30 to 31.67 min, and then dropped back to 1% B from 31.67 to 32.3 min. The respective nucleotides were collected by monitoring the absorbance at 254 nm. The collected samples from MONO Q were used in Ca^{2+}-release assays or NAADP binding assays without further treatment. The collected samples from AG MP-1 were neutralized to about pH 7.5 by the addition of 2 *M* Tris-base before use. The concentrations of the collected samples were determined using an extinction coefficient of 18,000 at 260 nm. In some cases, samples of thio-NADP and NADP purified once over the AG MP-1 were rechromatographed over the same system and neutralized as described above before use.

Materials

NADP, thio-NADP, NAAD, and trifluoroacetic acid were purchased from Sigma (St. Louis, MO). AG MP-1 resin was obtained from Bio-Rad (Hercules, CA). NAADP was enzymatically synthe-

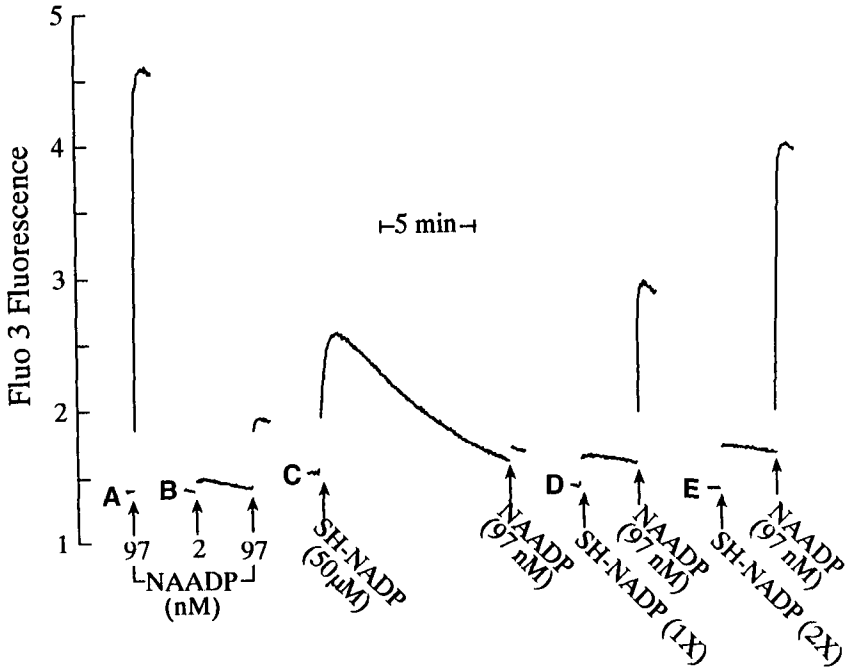

Fig. 1. The antagonistic effects of thio-NADP on Ca^{2+} release can be removed by purification. Ca^{2+} release was measured in sea-urchin egg homogenates. Unpurified thio-NADP (SH-NADP) was added to a final concentration as indicated. The same sample was also purified once (SH-NADP [1X]) or twice (SH-NADP [2X]) on an AG MP-1 column as described in the text. Also shown is the inhibitory effect of treating the egg homogenates with 2 n*M* NAADP for 2 min prior to challenging them with a saturating concentration of 97 n*M*.

sized from NADP and nicotinic acid using *Aplysia* ADP-ribosyl cyclase as previously described *(11)*. The MONO Q HR 5/5 column was from Pharmacia (Piscataway, NJ).

RESULTS AND DISCUSSION

A novel feature unique to the NAADP-sensitive Ca^{2+} release mechanism is that the agonist, NAADP itself, is a potent inactivator of the release mechanism even at subthreshold concentrations *(7,9)*. Figure 1 shows that pretreament of egg homogenates with 2 n*M* NAADP virtually eliminated the ability of 97 n*M* NAADP added 2 min later from releasing Ca^{2+} (Fig. 1, trace B), as compared to the control without pretreatment (trace A). Unpurified thio-NADP

Table 1
Effect of Various Substances on NAADP and cADPR-Induced Ca^{2+} Release

	Calcium release, %	
Antagonist	NAADP (1 μ*M*)	cADPR (1 μ*M*)
Control	100.0 ± 2.8	100.0 ± 10.0
NAADP (2 n*M*)	17.1 ± 2.6	98.3 ± 4.8
8-amino-cADPR (1 μ*M*)	100.4 ± 3.3	1.8 ± 0.8
Thio-NADP, unpurified (50 μ*M*)	1.8 ± 0.8	115.5 ± 0.8
Thio-NADP, 1X purified (50 μ*M*)	73.7 ± 1.3	113.8 ± 4.8
NADP, 1X purified (50 μ*M*)	23.0 ± 0.2	99.9 ± 3.3
NADP, 2X purified (50 μ*M*)	49.1 ± 8.9	107.0 ± 4.1
NAAD, unpurified (50 μ*M*)	54.9 ± 4.7	100.5 ± 3.0
NAAD, 1X purified (50 μ*M*)	74.6 ± 5.0	93.5 ± 6.1

Sea-urchin egg homogenates were treated with various antagonists for 3 min and challenged subsequently with either NAADP (1 μ*M*) or cADPR (1 μ*M*). Calcium release was normalized to that of the control without the antagonist treatment. Thio-NADP and NAAD were purified by HPLC (1x purified). NADP was either purified one (1x) or two times (2x) by HPLC. Values shown are mean ± SD of three determinations.

(50 μ*M*) induced submaximal Ca^{2+} release and desensitized the homogenate to subsequent challenge with 97 n*M* NAADP (trace C). However, this effect is likely due to an impurity in the commercial thio-NADP sample, since purification of the sample by anion-exchange chromatography on AG MP-1 (traces D, 1X purified) eliminated the Ca^{2+} release activity and substantially reduced the inhibition of NAADP-induced release. Repurification of the once purified thio-NADP by AG MP-1 resulted in further elimination of the ability of thio-NADP to inhibit NAADP-induced Ca^{2+} release (trace E, 2X purified). The twice-purified sample showed no Ca^{2+} release activity, and the minor inhibition seen in trace E is likely owing to residual contamination.

Table 1 shows quantitative measurements of the removal of the inhibitory effects of various commercial substances on the NAADP-sensitive Ca^{2+} release by HPLC purification. Pretreatment of sea-urchin egg microsomes with 2 n*M* NAADP inhibited subsequent Ca^{2+} release in response to 1 μ*M* NAADP by 83%, but had no effect on the cADPR-induced release. Conversely, a specific antagonist of cADPR, 8-amino-cADPR, showed no effect on the NAADP-induced release. Unpurified thio-NADP inhibited NAADP-induced Ca^{2+} release by more than 98%. After purification, the inhibition was

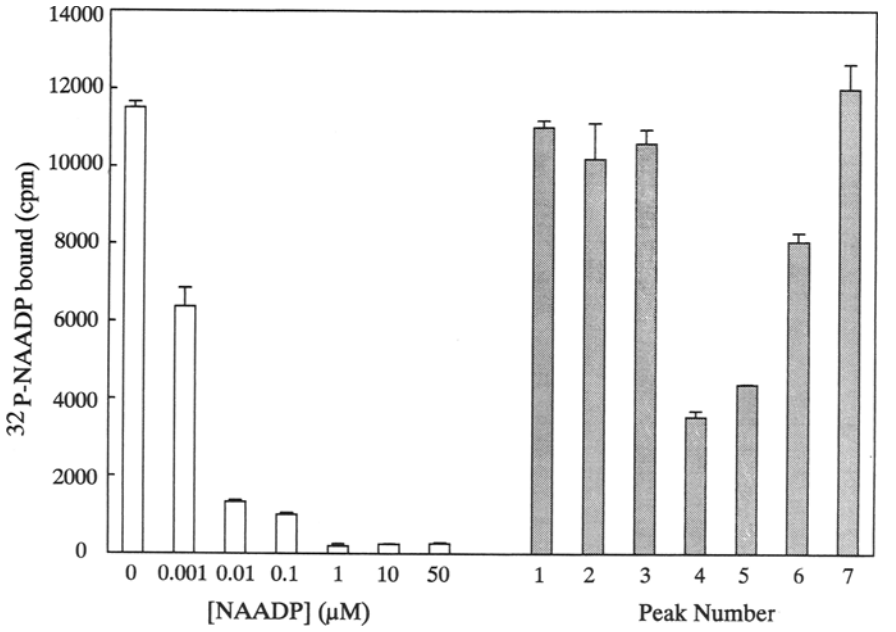

Fig. 2. Inhibition of ^{32}P-NAADP binding to egg microsomes by the impurities in the thio-NADP samples. The effects of various fractions on the ^{32}P-NAADP binding are shown in shaded bars. The competition by various concentrations of unlabeled NAADP is shown in open bars and the half-maximal effect is at about 1 n*M*.

reduced to about 26%. Similar to thio-NADP, unpurified NADP (data not shown) and NAAD both have strong inhibitory effects on the NAADP-induced Ca^{2+} release, which can likewise be removed by purification (Table 1). Indeed, the residual inhibition of the purified thio-NADP was no more than that produced by NADP or nicotinic acid adenine dinucleotide (NAAD) purified under the same conditions (Table 1). The inhibitory effects of thio-NADP, NADP, or NAAD are specific for the NAADP-induced Ca^{2+} release since none of them inhibited the cADPR-sensitive Ca^{2+} release.

It has previously been shown that ^{32}P-NAADP binds to specific sites present in sea-urchin egg microsomes, which can be competitively inhibited by nanomolar concentrations of unlabeled NAADP (ref. *9; see* Fig. 2). Unpurified thio-NADP and NADP likewise competed for the ^{32}P-NAADP binding sites in the egg microsomes with half-maximal inhibition occurring at 40 and 1 μ*M*, respectively (data not shown). When assayed at concentrations of 50 μ*M*, unpurified

Table 2
Effect of Purified Thio-NADP
and NADP on ^{32}P-NAADP Binding

	^{32}P-NAADP binding, %	
	Thio-NADP	NADP
Control	100.0	100.0
Stock (50 μ*M*)	44.2	3.0
Purified 1X (50 μ*M*)	82.8	51.2
Purified 2X (50 μ*M*)	77.2	84.8

Thio-NADP and NADP were purified once *(1X)* or twice *(2X)* on an AG MP-1 and tested at 50 μ*M* for inhibition of ^{32}P-NAADP binding to egg microsomes fractionated by Percoll gradient centrifugation. The control represents total binding without inhibitor. Values are average of duplicates.

thio-NADP and NADP inhibited binding by 66 and 97%, respectively (Table 2). Purification of thio-NADP once over AG MP-1 reduced this inhibition to about 17%. An additional purification of the purified thio-NADP did not change the binding appreciably. Similarly, purification of NADP reduced the inhibition to <15% (Table 2). The concentrations of purified thio-NADP and NADP that inhibited NAADP binding by 50% could not be determined because the purified stock solutions were not concentrated enough to allow binding to be studied over 100 μ*M*. This data strongly suggests that both thio-NADP and NADP are contaminated with a substance that inhibits NAADP binding.

The nature of the contaminant in thio-NADP was evaluated by purification using anion-exchange chromatography on a MONO-Q column that has greater resolving power than the AG MP-1 column. Figure 3 shows the elution profile of 800 nmol of thio-NADP chromatographed on MONO Q and eluted with a triethylammonium bicarbonate gradient as described under Materials and Methods. It is apparent that the sample contained multiple contaminants. Fractions corresponding to various peaks labeled 1–7 were collected and examined for their ability to compete with ^{32}P-NAADP in the binding assay (Fig. 2). Thio-NADP, the major peak, had minimal inhibition on ^{32}P-NAADP-binding as shown in Table 2. The largest inhibition was seen with fraction 4. This fraction had an elution time of about 24 min, very close to the elution time of authentic

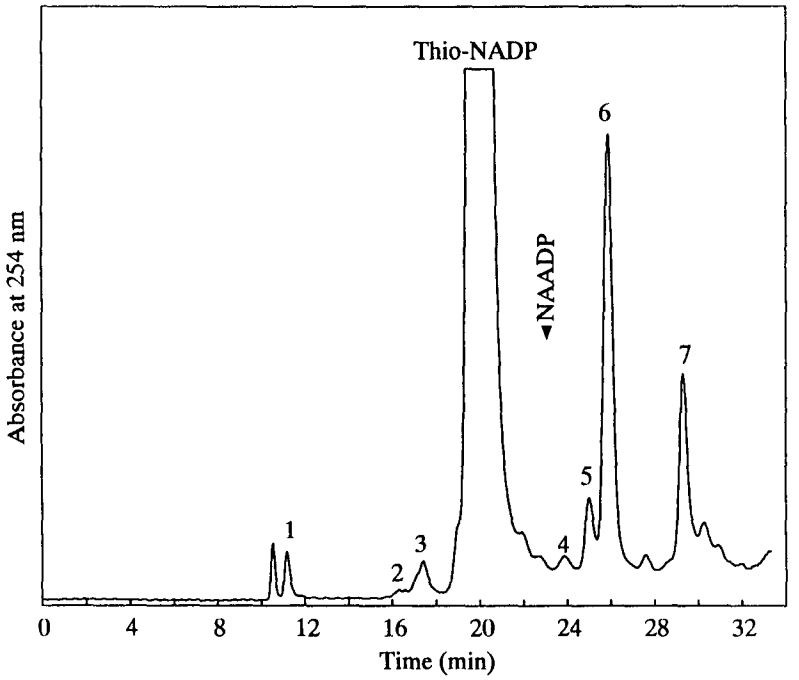

Fig. 3. Purification of thio-NADP on a Mono-Q column. Multiple contaminant peaks are apparent in the thio-NADP sample. Seven (numerically labeled) fractions were collected and tested for inhibition on ^{32}P-NAADP binding to egg microsomes. The retention time of authentic NAADP on this column is about 23 min and is indicated by an arrowhead.

NAADP (indicated on the figure). Progressively less inhibition on ^{32}P-NAADP-binding was seen in fractions 5 and 6, suggesting the residual effects were due to trailing off of the contaminant that was mainly collected in fraction 4. The similarity of the elution time of the inhibitory contaminant with NAADP suggests that it is NAADP itself. The amount of the contaminating NAADP estimated by comparing the inhibitory effects of fractions 4–6 with that of authentic NAADP, also shown in Fig. 2, is 0.012%. An unpurified solution of thio-NADP at a concentration of 50 μ*M* would contain 6 n*M* NAADP. We have previously shown that 30 n*M* NAADP induces half-maximal Ca^{2+} release from egg microsomes *(1,9)*. The submaximal Ca^{2+} release induced by 50 μ*M* unpurified thio-NADP shown in Fig. 1, trace C, is consistent with a contamination of NAADP somewhere in the range of 10 n*M*. The ability of 50 μ*M* unpurified thio-NADP to inhibit NAADP binding by 56% (Table 2) is also consistent

with contamination by NAADP in the low nanomolar range as half maximal inhibition of binding is observed between 1 and 10 n*M* NAADP (ref. *1; see* Fig. 2). Therefore, these results indicate that the inhibitory effects of thio-NADP on both Ca^{2+} release and ^{32}P-NAADP-binding can be accounted for if thio-NADP were contaminated with this level of NAADP.

The fact that nanomolar concentrations of NAADP can self-inactivate the Ca^{2+}-release mechanism makes the search of other specific antagonists extremely difficult. Mistreatment of samples can potentially produce sufficient NAADP to make them appear as antagonists. For example, it has been shown that alkaline treatments can easily convert NADP to NAADP *(6)*. Indeed, sufficient amounts of contaminating NAADP are usually present in commercial NADP samples to produce Ca^{2+}-release activity. It was this observation that had led to the discovery of NAADP *(6)*. In any case, results in this study show that thio-NADP is no more an antagonist than unpurified NADP or NAAD, making its use of questionable value.

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