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Nicotinic Acid Adenine Dinucleotide 2'-Phosphate (NAADP) Binding Proteins in T-Lymphocytes

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Abstract

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a messenger that regulates calcium release from intracellular acidic stores. Although several channels, including two-pore channels (TPC), ryanodine receptor (RyR) and mucolipin (TRP-ML1) have been implicated in NAADP regulation of calcium signaling, the NAADP receptor has not been identified. In this study, the photoaffinity probe, [³²P]-5-azido-NAADP ([³²P]-5-N₃-NAADP), was used to study NAADP binding proteins in extracts from NAADP responsive Jurkat T-lymphocytes. [³²P]-5-N₃-NAADP photolabeling of Jurkat S100 cytosolic fractions resulted in the labeling of at least ten distinct proteins. Several of these S100 proteins, including a doublet at 22/23 kDa and small protein at 15 kDa displayed selectivity for NAADP as the labeling was protected by inclusion of unlabeled NAADP, whereas the structurally similar NADP required much higher concentrations for protection. Interestingly, the labeling of several S100 proteins (60, 45, 33 and 28 kDa) was stimulated by low concentrations of unlabeled NAADP, but not by NADP. The effect of NAADP on the labeling of the 60 kDa protein was biphasic, peaking at 100 nM with a five-fold increase and displaying no change at 1 μM NAADP. Several proteins were also photolabeled when the P100 membrane fraction from Jurkat cells was examined. Similar to the results with S100, a 22/23 kDa doublet and a 15 kDa protein appeared to be selectively labeled. NAADP did not increase the labeling of any P100 proteins as it did in the S100 fraction. The photolabeled S100 and P100 proteins were successfully resolved by two-dimensional gel electrophoresis. [³²P]-5-N₃-NAADP photolabeling and two-dimensional electrophoresis should represent a suitable strategy in which to identify and characterize NAADP binding proteins.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Keywords

NAADP; Jurkat T Cells; Photoaffinity Labeling; [^{32}P]5-N₃-NAADP

INTRODUCTION

Nicotinic acid adenine dinucleotide phosphate (NAADP) is the most potent Ca^{2+} mobilizing second messenger known so far. Lee and Aarhus discovered NAADP as a contaminant in commercial nicotinamide adenine dinucleotide phosphate (NADP) preparations (Lee and Aarhus, 1995). In the meantime, the Ca^{2+} mobilizing activity of NAADP has been demonstrated in many different cell types [reviewed in citations (Lee, 2005; Fliegert et al., 2007; Galione et al., 2011; Lee, 2011)]. To be defined as second messenger the following requirements must be fulfilled: (i) intracellular formation upon stimulation of cells by an extracellular (first) messenger, (ii) subsequent transmission of the signal by the second messenger, and (iii) degradation of the second messenger to terminate the signal. The first and the last point refer to metabolism of NAADP and will not be covered here; however, the molecular mechanism of signal transmission by NAADP is central to this article.

What is known about the mechanism of action of NAADP? Work in sea urchin eggs very early showed that in Ca^{2+} release experiments there was no cross-desensitization between d-*myo*-inositol 1,4,5-trisphosphate (IP_3), cyclic ADP-ribose (cADPR), or NAADP (Lee and Aarhus, 1995). Furthermore, the Ca^{2+} store sensitive to NAADP was distinct from the endo/sarcoplasmic reticulum (ER/SR) (Lee and Aarhus, 2000; Churchill et al., 2002). These data indicated that NAADP may not act as described for the 'classical' Ca^{2+} mobilizing second messenger second messengers IP_3 and cADPR via release of Ca^{2+} from the ER/SR, but by mediating Ca^{2+} release from acidic stores, probably via a new NAADP-sensitive channel (Lee and Aarhus, 2000; Churchill et al., 2002).

In contrast to these findings in sea urchin eggs, evidence for involvement of ryanodine receptors (RyR) was published. Using microsomes from heart (Mojzisova et al., 2001) or highly purified RyR preparations from skeletal muscle (Hohenegger et al., 2002) reconstituted in lipid planar bilayers, an increase in the channel open probability upon NAADP addition to the cis-chamber was observed. Moreover, NAADP evoked Ca^{2+} release from nuclei prepared from pancreatic acinar cells also suggested involvement of RyR (Gerasimenko et al., 2003a; Gerasimenko et al., 2003b). In pancreatic acinar cells, the situation may be more complex: inhibitors of RyR blocked Ca^{2+} release evoked by NAADP in both acidic organelles and the ER indicating localization of RyR on both types of stores (Gerasimenko et al., 2006a; Gerasimenko et al., 2006b).

A further candidate for NAADP sensitive Ca^{2+} channels was discovered by Li and coworkers who fused the lysosomally located transient receptor potential channel, subtype mucolipin 1 (TRP-ML1) into lipid planar bilayers and observed channel opening evoked by NAADP (Zhang et al., 2009). Moreover, in fibroblasts not expressing TRP-ML1, NAADP did not evoke Ca^{2+} release; however, expression of TRP-ML1 in such cells restored the effect of NAADP (Zhang et al., 2011). In contrast, Yamaguchi et al., found no obvious role for TRP-ML1 in NAADP evoked Ca^{2+} signaling (Yamaguchi et al., 2011).

In 2009, two-pore channels (TPC) were published as strong candidates for the NAADP sensitive Ca^{2+} channels located in acidic stores (Brailoiu et al., 2009; Calcraft et al., 2009; Zong et al., 2009). TPCs belong to the voltage-sensitive ion channel super-family (Calcraft et al., 2009). In a number of cell types, Ca^{2+} signals mediated by NAADP are increased when TPCs are over-expressed (Calcraft et al., 2009; Zong et al., 2009; Brailoiu et al., 2010; Dionisio et al., 2011; Ogunbayo et al., 2011; Pereira et al., 2011). Moreover, gene silencing of TPCs by either siRNA or gene knockout resulted in decreased or absent effects of NAADP (Brailoiu et al., 2009; Calcraft et al., 2009).

The human T-lymphoma cell line Jurkat was one of the first mammalian cell types in which NAADP evoked Ca^{2+} release was observed (Berg et al., 2000). In a number of reports we demonstrated that

- i. endogenous NAADP was formed upon stimulation of the T cell receptor (TCR)/CD3 complex (Gasser et al., 2006),
- ii. RyR are necessary for both global and local Ca^{2+} release (Langhorst et al., 2004; Dammermann and Guse 2005), and
- iii. NAADP evoked Ca^{2+} release was not blocked by the V-ATPase inhibitor bafilomycin A1 (Steen et al., 2007).

In addition, we demonstrated that a specific antagonist of NAADP, termed BZ194, effectively inhibited re-activation of post-activated effector T cells (Dammermann et al., 2009), thereby demonstrating that NAADP signaling is not an exotic event in a T-lymphoma line, but is of pathophysiological importance in autoimmunity of the brain (Cordiglieri et al., 2010).

Recently, some of the authors developed NAADP analogues with modifications in the 4- and 5-position of the nicotinic acid moiety of NAADP (Jain et al., 2010). One of these analogs, 5-azido-NAADP (5- N_3 -NAADP) activated Ca^{2+} release and competed with ^{32}P -NAADP in a competition binding assay and was further developed for use as a photoaffinity probe to detect NAADP receptors. The advantages of this newly developed photoaffinity probe include high affinity as compared to NAADP itself, and in contrast to classical labeling techniques, the ability to covalently label proteins. The latter can be visualized using one- or two-dimensional SDS-PAGE techniques, respectively. Using radioactively labeled [^{32}P]5- N_3 -NAADP, binding proteins for NAADP (NAADP-BP) were characterized by photoaffinity labeling in sea urchin egg homogenates (Walseth et al., 2012). The labeled probe was found to photolabel several proteins that possessed properties similar to the high affinity NAADP binding previously studied in this system in terms of affinity, selectivity and pseudo-irreversibility in the presence of high potassium (Walseth et al., 2012). The [^{32}P]5- N_3 -NAADP photoprobe has also been used in mammalian cell lines and mouse pancreas (Lin-Moshier et al., 2012). The [^{32}P]5- N_3 - NAADP photoprobe was able to detect several proteins that bound NAADP selectively and with high affinity and appeared to be distinct from putative NAADP regulated channels discussed above (RYR, TRPML1 and TPCs) (Lin-Moshier et al., 2012; Walseth et al., 2012). Lin- Moshier et al., suggested that a critical reassessment of the mechanisms involved in NAADP-induced calcium mobilization was needed (Lin-Moshier et al., 2012). Here, we applied this approach to protein

preparations from Jurkat T cells and extend the characterization of NAADP-BP using two-dimensional SDS-PAGE.

MATERIALS AND METHODS

Materials

HEPES, NAD, NAAD, NADP, NMN, nicotinic acid, nicotinamide, DTT, CHAPS, dodecyl-maltoside, urea and thiourea were from Sigma. Laemmli sample buffer, 12.5% Criterion acrylamide gels and 12.5% IPG+1 gels and AG MP-1 resin were obtained from Bio-Rad. Perfect-FOCUS kits were from G-Biosciences. Reagents for two-dimensional electrophoresis, including, Immobiline Dry Strip Gels (13 cm, pH 3–10), Destreak Hydration solution and IPG Buffer pH 3–10 were obtained from GE Healthcare Life Sciences. SimplyBlue Safe Stain was obtained from Invitrogen. Recombinant human NAD kinase was purchased from Alexis. Complete protease inhibitor tablets were from Roche. [^{32}P]-NAD (800Ci/mmol) was from Perkin-Elmer. NAADP, 5N₃-NAADP and 5N₃-nicotinic acid were prepared as previously described (Jain et al., 2010).

Synthesis of [^{32}P] – 5N₃-NAADP

[^{32}P]-5N₃-NAADP was synthesized from [^{32}P]-NAD as described previously (Walseth et al., 2012).

Preparation of Protein Fractions from Jurkat T Lymphocytes

Jurkat T cells were cultured as previously described (Schweitzer et al., 2001). Cytosolic (S100) and 100000×g pellet (P100) fractions were prepared as published (Schweitzer et al., 2001). The S100 and P100 fractions were stored at –80 °C in 20 mM Hepes, 110 mM NaCl, pH 7.3 containing protease inhibitors.

Purification of Nucleotides by Semipreparative HPLC

The purification system used to remove any NAADP contaminating commercial sources of NADP, NAAD and NAD used in competition experiments has been described (Walseth et al., 2012). The purification of these nucleotides just prior to use is necessary since NAADP was originally discovered as a contaminant in commercial NADP (Lee and Aarhus, 1995).

Photoaffinity Labeling of Jurkat Subcellular Fractions with [^{32}P]-5N₃-NAADP

Jurkat S100 or P100 fractions were diluted to 1 mg/ml with 20 mM Hepes, 110 mM NaCl, pH 7.3. The standard photolabeling protocol was as follows. The diluted fractions (40 μl , 40 μg protein) were incubated in a total volume of 50 μl with 2–8 nM [^{32}P]-5N₃-NAADP for 60 minutes at 4 °C. Unlabeled competitors were made to the final concentrations indicated in the figure legends. UV photolysis was accomplished by placing the samples on a sheet of saran wrap stretched over a block of ice and irradiated with UV light (10^{15} quanta/s) for 2 min in a Rayonet Photochemical Reactor (Southern New England Ultraviolet Company). After photolysis, 40 μl of the photolyzed samples were recovered and 10 μl of 5 × Laemmli SDS sample buffer was added. The samples were allowed to incubate at room temperature for 30 minutes and then subjected to SDS-polyacrylamide gel electrophoresis using 12.5%

Criterion acrylamide gels. The gels were stained with SimplyBlue Safe Stain, destained and air-dried between two sheets of cellophane. Analysis of photolabeling was accomplished by exposing the dried gels to MP storage phosphor screens (Packard Instruments) for 24–96 hours. The phosphor screens were developed using a Cyclone Phosphor Imager (Packard Instruments). Quantification of the phosphor images was performed with OptiQuant software (Version 3.0, Packard Instruments).

Two-Dimensional Separation of NAADP-BP by Electrophoresis

Photoaffinity labeling was performed as described above. The photolabeled samples were precipitated using Perfect-FOCUS kits using the manufacturer's protocol. The resulting precipitates were dissolved in 20 μ l of lysis buffer (4% CHAPS, 7 M urea, 2 M thiourea, 1% dodecyl-maltoside). The samples were incubated on ice for 60 minutes with occasional mixing. The following were then added to the sample (2.5 μ l 1 M DTT, 1.25 μ l IPG Buffer pH 3–10 and 226 μ l of Destreak Hydration Solution) and incubated for 15 minutes at room temperature. The sample was then loaded onto a 13 cm Immobiline Dry Strip Gel pH 3–10 and allowed to hydrate for 1 hour. The strip was focused for 17 hours (17500V-hr). The focused strip was equilibrated with 75 mM Tris-HCl, pH 8.8, 6 M urea, 29.3% glycerol, 2% SDS, 0.002% bromophenol blue and the second dimension run on 12.5% IPG+1 Criterion gel. The gels were stained and dried as described above.

RESULTS

Photoaffinity labeling of the Jurkat cytosolic S100 fraction using [32 P]–5- N_3 -NAADP resulted in labeling of 8 single bands (termed NAADP-BP 1 to 7, 10) and a doublet (termed NAADP-BP 8/9; Fig. 1(A)). Selectivity was assessed by comparing the effect of purified NAADP or NADP between 1 and 100 nM (Fig. 1(A)) and structurally related compounds (Fig. 1(B)) on the labeling. Densitometric analyses of each labeled NAADP-BP is shown in Figure 1(C). The labeling of the NAADP BP 8/9 at approximately 22/23 kDa was protected better by lower concentrations of NAADP than with NADP, suggesting it was selective for NAADP (Figs. 1(A and C)). The decrease of labeling of NAADP-BP 8/9 was half-maximal at 4.2 nM NAADP and 191 nM NADP (Fig. 1(C)). NAADP BP 6 and 10, minor bands at about 31 and 15 kDa, respectively, also appeared to have some selectivity for NAADP. Unexpectedly, the intensity of NAADP-BP 1, 2, 5 and 7 increased with increasing concentrations of competing unlabeled NAADP (Fig. 1(A)), while competition with unlabeled NADP up to 30 nM did not change the labeling of any NAADP-BP (Fig. 1(A)). Densitometric analysis revealed over 5-fold increase of labeling of NAADP-BP 1 (approx. 60 kDa) at 100 nM NAADP with an EC_{50} of approx. 10 nM NAADP, whereas NADP failed to increase labeling (Fig. 1(C)). Concentrations of NAADP above 1 μ M did not increase the labeling of NAADP BP 1. NAADP BP 3, 7 appeared to be more selective for NADP and were not considered further. Figure 1(B) shows that photolabeling of Jurkat S100 in the presence of structurally related nucleotides such as nicotinic acid, nicotinamide, NAAD, NAD and NMN did not significantly affect the photolabeling of S100 NAADP-BPs by [32 P]–5- N_3 -NAADP, indicating that the labeling is highly selective.

We also examined [^{32}P]-5- N_3 -NAADP photoaffinity labeling in the Jurkat P100 membrane fraction (Fig. 2(A)). Photolabeling of P100 resulted in labeling of 7 single bands (termed NAADP-BP I to VII). Densitometric analyses are shown in Figure 2(B). Unlabeled NAADP in the nanomolar range prevented labeling of NAADP-BP I, IV, VI and VII (Fig. 2(A)); however, NADP also prevented the labeling of NAADP-I and IV at the same concentration (100 nM; Fig. 2(A)). No P100 proteins labeled by [^{32}P]-5- N_3 -NAADP were stimulated by addition of unlabeled NAADP as shown above for S100 proteins. P100 NAADP BP VI and VII appear to be the same as S100 NAADP BP 8/9 and 10, respectively. Densitometric analysis of the membrane fraction proteins NAADP-BP VI and VII showed a similar concentration-response curve for competition by unlabeled NAADP as compared to NAADP-BP 8/9 and 10 (Fig. 2(B)).

Identification of proteins labeled selectively by [^{32}P]-5- N_3 -NAADP in the S100 and P100 will be necessary in order to fully understand the mechanism(s) by which NAADP regulates calcium signaling. We examined two-dimensional gel electrophoresis (2D gels) as a first step in the identification of these proteins. Photolabeled proteins were resolved using isoelectric focusing (IEF) between pH 3 and pH 10 in the first dimension and a 12.5% SDS-PAGE in the second dimension. Figures 3 and 4 show 2D gels of S100 and P100 proteins photoaffinity labeled in the absence (panel A) or presence of 500 nM NAADP (panel B), respectively. In Figures 3 and 4 labeled protein spots where labeling decreased in the presence of 500 nM NAADP are circled in red and those enhanced by 500 nM NAADP are circled in green. The NAADP-BP 8/9 doublet was separated by IEF into 2 spots with isoelectric points (IEP) at just under pH 7. The small molecular weight NAADP-BP 10 was separated into 4 spots with IEP at between 6 and 8. As indicated above for the 1-dimensional SDS-PAGE, the unexpected increase in NAADP-BP upon increasing NAADP concentrations was also observed. Labeling of NAADP-BP 1 was significantly increased and was detected in at least 5 individual spots between pH 6 and pH 7.

Two-dimensional gel electrophoresis of P100 protein, carried out as above for S100 protein, resulted in specific labeling of NAADP-BP I which appeared as a single protein at about 75 kDa with an IEP of approx. 5 (Fig. 4). NAADP-BP II was also specifically labeled with several close spots between pH 6 and 6.8; interestingly, this specific labeling was not observed in the one-dimensional separation (NAADP-BP II in Fig. 1(B)). As in the one-dimensional gel labeling of NAADP-BP III was not prevented by unlabeled NAADP. NAADP-BP IV was separated by IEF into several spots that were all protected from photolabeling by unlabeled NAADP (Fig. 4). The 2 major spots had IEPs just above pH 6, but weaker spots at just below pH 6 and between pH 7 and 8 were also observed. Another weakly labeled and poorly resolved set of NAADP-BP IV was observed between pH 7 and 9 (Fig. 4). Another 2 spots that were not resolved in the 1-dimensional gels (Fig. 1(B)) appeared at a molecular mass of about 31 kDa and IEP just below pH 6 and about pH 7.5, respectively (Fig. 4, termed IVb here). NAADP-BP V had a molecular mass of about 29 kDa (see Figs. 1(B) and 4). Three spots with specific labeling were separated at approximately pH 4.3, 5.0 and 5.5 (Fig. 4). NAADP-BP VI at 24 kDa showed an IEP of about 6.5 (Fig. 4). The low molecular mass NAADP-BP VII was detected as a broad and relatively weak spot with an IEF < 4 (Fig. 4).

DISCUSSION

In this report we describe characterization of NAADP-BPs by photoaffinity labeling with [^{32}P]-5N $_3$ -NAADP in Jurkat T cells, a cell type in which NAADP signaling has been reported repeatedly (Berg et al., 2000; Steen et al., 2007). Specific binding proteins in the molecular weight range from approximately 15 to 75 kDa were detected in the S100 cytosolic fraction. In the presence of physiological concentrations of unlabeled NAADP both increases and decreases of labeling of the NAADP-BPs were observed. In membrane fractions a set of proteins with similar molecular masses was detected. In this fraction unlabeled NAADP only decreased labeling of NAADP-BPs. NAADP-BPs were further characterized by 2-dimensional IEF/SDS-PAGE.

Photoaffinity labeling as an approach to characterize and ultimately to identify protein(s) interacting with a specific ligand has been successfully used in various fields (Dorman and Prestwich, 2000; Lamos et al., 2006). However, Robinette and colleagues suggested a number of checkpoints to control such experiments (Robinette et al., 2006). Potential pitfalls may be related to

- i. interference of the photo-labile moiety during binding of the ligand to its receptor,
- ii. largely decreased affinity of the photoaffinity label, and
- iii. chemical stability of the photoaffinity label.

Since the chemical stability of nucleotides carrying azido groups has been reported (Weber and Beck-Sickinger, 1997), altered affinity and perhaps altered binding kinetics of [^{32}P]-5N $_3$ -NAADP may be of greater importance here. While modification of the 4-position of the nicotinic acid moiety of NAADP resulted in an at least 235-fold higher EC $_{50}$ for Ca $^{2+}$ release, some of the 5-substituted analogues were almost equipotent to NAADP (Jain et al., 2010). 5-N $_3$ -NAADP, a potential photoaffinity label for NAADP-BPs, was only 90-fold weaker in Ca $^{2+}$ release (Jain et al., 2010). More importantly, the 5-substituted analogues displaced [^{32}P]NAADP binding in sea urchin egg homogenates in an almost similar fashion as NAADP, e.g., the IC $_{50}$ decreased only 11-fold for 5-N $_3$ -NAADP (Jain et al., 2010). Thus, though there is an obvious small interference of the 5-azido group with NAADP's affinity to its putative receptor, 5-N $_3$ -NAADP in general appears an attractive tool for photoaffinity labeling of NAADP-BP(s).

Robinette et al., further pointed out that during the labeling process itself, the photo-activated state of the photoprobe should be very short to allow specific labeling, but not random labeling of bystander proteins (Robinette et al., 2006). Since the NAADP-BPs defined so far have not yet been molecularly identified, it is very difficult to judge the photoaffinity label in this regard. Finally, proteolysis of protein during the labeling process must be prevented to ensure correct mass determination during downstream analytical procedures such as SDS-PAGE (Robinette et al., 2006). Specific labeling of only 3 bands in sea urchin egg homogenates by [^{32}P]-5N $_3$ -NAADP recently demonstrated that obviously proteolysis secondary to labeling does not appear to be a big problem (Walseth et al., 2012). However, given the fact that most the candidate Ca $^{2+}$ channels suggested to be involved in NAADP mediated Ca $^{2+}$ release, TPCs or RyRs, are characterized by a larger molecular

mass than the NAADP-BPs described so far (including the data presented in this report), we cannot fully exclude that proteolytic fragments of these channels might be among the various NAADP binding proteins detected here. However, no changes in the [^{32}P]- 5N_3 -NAADP photolabeling patterns were observed in pancreatic samples from TPC1 and TPC2 knockout mice compared to wild type, arguing that the photolabeled proteins are not proteolytic fragments derived from TPCs (Lin-Moshier et al., 2012). Recently, it was shown that endogenous TRP-ML1, a lysosomal monovalent cation channel, is proteolytically cleaved into smaller (approx. 40–60 kD), non-active forms (Kiselyov et al., 2005). Thus, we cannot rule out that some of the labeled protein bands detected in Jurkat P100 are TRP-ML1. Interestingly, Lin-Moshier et al., (Lin-Moshier et al., 2012) reported that in photoaffinity-labeled protein samples from SKBR3 cells overexpressing TPCs no changes in the labeling pattern were observed. Furthermore, in protein samples from pancreas tissue from TPC1^{-/-} or TPC2^{-/-} mice the labeling pattern remained the same suggesting that TPCs do not bind NAADP directly. In P100 membranes from Jurkat T cells specific labeling of RyR has not yet been observed (Fig. 2(A)) indicating that this may also hold true for RyR. Molecular identification of the individual labeled proteins is required to finally answer these questions.

In the previous report by Lin-Moshier et al., (Lin-Moshier et al., 2012) in three different mammalian cell systems, (the cell lines SKBR 3 and HEK293 and in mouse pancreatic samples (17,000 × g supernatant)), different NAADP BPs were detected. Based on 2 criteria,

- i. competition of labeling at low NAADP concentrations and
- ii. specificity over NADP in such competition experiments revealed that a 22/23 kD doublet was the most promising candidate arising from all 3 cell systems (Lin-Moshier et al., 2012).

Interestingly, we also detected the 22/23 kD doublet in Jurkat S100 (NAADP BP 8/9) and P100 fractions (NAADP BP VI) and, similar to Lin-Moshier et al., (Lin-Moshier et al., 2012) observed a 45-fold specificity for NAADP over NADP (Fig. 1(C)). In addition to the 22/23 kD doublet, a smaller protein of approx. 15 kD also showed somewhat elevated specificity over NADP at 100 nM in Jurkat T cells (Figs. 1(A and C)). A similarly labeled band was neither detected in the other mammalian system (Lin-Moshier et al., 2012) nor in sea urchin egg homogenates (Walseth et al., 2012). Interestingly, four bands exhibited an unusual behavior in the competition experiments. Labeling of NAADP BPs 1, 2, 4, and 5 increased with increasing NAADP in the range of 1 to 100 nM while NADP had little or no effect (Figs. 1(A, C)). Though the molecular mechanism underlying this observation is not entirely clear one may speculate that these Jurkat T cell proteins are low affinity/high capacity NAADP binding proteins. The concentration of [^{32}P]- 5N_3 -NAADP used (2 nM) would be below the binding affinity of the protein and not be high enough to bind these NAADP binding sites to any appreciable extent. As soon as higher unlabeled NAADP concentrations are employed, more [^{32}P]- 5N_3 -NAADP binds, due to the concentration of total NAADP increasing towards the binding affinity of the protein. Further characterization of these NAADP binding proteins in terms of their binding affinities and other properties will be necessary before the mechanism by the increases in photolabeling occur can be understood.

Identification of the photolabeled NAADP BPs will be critical in gaining a full understanding of NAADP action. We demonstrate that two-dimensional gel electrophoresis may be a valuable tool in resolving the photoaffinity labeled NAADP BPs. However, there are several drawbacks that need to be considered. First, the efficiency of [^{32}P]-5N₃-NAADP photolabeling is unknown and is most likely low. Second, photoaffinity labeling introduces a highly charged molecule that will change the isoelectric point of the protein, causing the separation of the cross-linked proteins from unmodified proteins on the first dimension of the two-dimensional system. Given these limitations other methods to enrich the photolabeled NAADP BPs will be necessary for identification. Immobilized metal affinity chromatography techniques commonly used to enrich phosphopeptide (Thingholm et al., 2009) that would take advantage of the phosphate containing moiety crosslinked to the protein or development of a bifunctional probe (Best, 2009) are possibilities.

CONCLUSION

Taken together we confirm expression of the 22/23 kDa doublet NAADP BP with high affinity binding and high specificity over NADP in Jurkat T-lymphocytes, as shown previously for 2 other mammalian cell lines and pancreatic tissue. As novel and highly interestingly observation we present specific NAADP BPs in which photoaffinity labeling was increased, not decreased, over the physiological concentration range of NAADP (3 to 100 nM). Though the underlying molecular mechanism of this behavior currently remains unclear we speculate that these NAADP BPs might be important factors of conveying the NAADP signal to the NAADP sensitive channel in T cells. Thus, in addition to SKBR3 cells, HEK cells and mouse pancreas, Jurkat T cells may constitute another example fitting into the unifying hypothesis for NAADP action published recently (Guse, 2012). As the central aspect of the unifying hypothesis NAADP needs to bind to specific binding proteins to modulate different ion channels, e.g., RyRs, TPCs, TRP-ML1 and perhaps others, too.

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List of Abbreviations

NAADP	nicotinic acid adenine dinucleotide 2'-phosphate
RyR	ryanodine receptor(s)
TPC	two-pore channel
TRP	transient receptor potential
wt	wild type
IEP	isoelectric point

REFERENCES

- Berg I, Potter BV, Mayr GW, Guse AH. Nicotinic acid adenine dinucleotide phosphate (NAADP(+)) is an essential regulator of T-lymphocyte Ca(2+)-signaling. *J. Cell Biol.* 2000; 150:581–588. [PubMed: 10931869]
- Best MD. Click chemistry and bioorthogonal reactions: Unprecedented selectivity in the labeling of biological molecules. *Biochemistry.* 2009; 48:6571–6584. [PubMed: 19485420]
- Brailoiu E, Churamani D, Cai X, Schrlau MG, Brailoiu GC, Gao X, Hooper R, Boulware MJ, Dun NJ, Marchant JS, Patel S. Essential requirement for two-pore channel 1 in NAADP-mediated calcium signaling. *J. Cell Biol.* 2009; 186:201–209. [PubMed: 19620632]
- Brailoiu E, Rahman T, Churamani D, Prole DL, Brailoiu GC, Hooper R, Taylor CW, Patel S. An NAADP-gated two-pore channel targeted to the plasma membrane uncouples triggering from amplifying Ca2+ signals. *J. Biol. Chem.* 2010; 285:38511–38516. [PubMed: 20880839]
- Calcraft PJ, Ruas M, Pan Z, Cheng X, Arredouani A, Hao X, Tang J, Rietdorf K, Teboul L, Chuang KT, Lin P, Xiao R, Wang C, Zhu Y, Lin Y, Wyatt CN, Parrington J, Ma J, Evans AM, Galione A, Zhu MX. NAADP mobilizes calcium from acidic organelles through two-pore channels. *Nature.* 2009; 459:596–600. [PubMed: 19387438]
- Churchill GC, Okada Y, Thomas JM, Genazzani AA, Patel S, Galione A. NAADP mobilizes Ca(2+) from reserve granules, lysosome-related organelles, in sea urchin eggs. *Cell.* 2002; 111:703–708. [PubMed: 12464181]
- Cordiglieri C, Odoardi F, Zhang B, Nebel M, Kawakami N, Klinkert WE, Lodygin D, Luhder F, Breunig E, Schild D, Ulaganathan VK, Dornmair K, Dammermann W, Potter BV, Guse AH, Flugel A. Nicotinic acid adenine dinucleotide phosphate-mediated calcium signalling in effector T cells regulates autoimmunity of the central nervous system. *Brain.* 2010; 133:1930–1943. [PubMed: 20519328]
- Dammermann W, Guse AH. Functional ryanodine receptor expression is required for NAADP-mediated local Ca2+ signaling in T-lymphocytes. *J. Biol. Chem.* 2005; 280:21394–21399. [PubMed: 15774471]
- Dammermann W, Zhang B, Nebel M, Cordiglieri C, Odoardi F, Kirchberger T, Kawakami N, Dowden J, Schmid F, Dornmair K, Hohenegger M, Flugel A, Guse AH, Potter BV. NAADP-mediated Ca2+ signaling via type 1 ryanodine receptor in T cells revealed by a synthetic NAADP antagonist. *Proc. Natl. Acad. Sci. USA.* 2009; 106:10678–10683. [PubMed: 19541638]
- Dionisio N, Albarran L, Lopez JJ, Berna-Ero A, Salido GM, Bobe R, Rosado JA. Acidic NAADP-releasable Ca(2+) compartments in the megakaryoblastic cell line MEG01. *Biochim. Biophys. Acta.* 2011; 1813:1483–1494. [PubMed: 21601596]
- Dorman G, Prestwich GD. Using photolabile ligands in drug discovery and development. *Trends in Biotechnology.* 2000; 18:64–77. [PubMed: 10652511]
- Fliegert R, Gasser A, Guse AH. Regulation of calcium signalling by adenine-based second messengers. *Biochem. Soc. Trans.* 2007; 35:109–114. [PubMed: 17233614]
- Galione A, Parrington J, Funnell T. Physiological roles of NAADP-mediated Ca2+ signaling. *Sci. China Life Sci.* 2011; 54:725–732. [PubMed: 21786195]
- Gasser A, Glassmeier G, Fliegert R, Langhorst MF, Meinke S, Hein D, Kruger S, Weber K, Heiner I, Oppenheimer N, Schwarz JR, Guse AH. Activation of T cell calcium influx by the second messenger ADP-ribose. *J. Biol. Chem.* 2006; 281:2489–2496. [PubMed: 16316998]
- Gerasimenko J, Maruyama Y, Tepikin A, Petersen OH, Gerasimenko O. Calcium signalling in and around the nuclear envelope. *Biochem. Soc. Trans.* 2003a; 31:76–78. [PubMed: 12546657]
- Gerasimenko JV, Flowerdew SE, Voronina SG, Sukhomlin TK, Tepikin AV, Petersen OH, Gerasimenko OV. Bile acids induce Ca2+ release from both the endoplasmic reticulum and acidic intracellular calcium stores through activation of inositol trisphosphate receptors and ryanodine receptors. *J. Biol. Chem.* 2006a; 281:40154–40163. [PubMed: 17074764]
- Gerasimenko JV, Maruyama Y, Yano K, Dolman NJ, Tepikin AV, Petersen OH, Gerasimenko OV. NAADP mobilizes Ca2+ from a thapsigargin-sensitive store in the nuclear envelope by activating ryanodine receptors. *J. Cell Biol.* 2003b; 163:271–282. [PubMed: 14568993]

- Gerasimenko JV, Sherwood M, Tepikin AV, Petersen OH, Gerasimenko OV. NAADP, cADPR and IP₃ all release Ca²⁺ from the endoplasmic reticulum and an acidic store in the secretory granule area. *J. Cell Sci.* 2006b; 119:226–238. [PubMed: 16410548]
- Guse AH. Linking NAADP to ion channel activity: A unifying hypothesis. *Sci. Signal.* 2012; 5:pe18. [PubMed: 22534131]
- Hohenegger M, Suko J, Gscheidlinger R, Drobny H, Zidar A. Nicotinic acid-adenine dinucleotide phosphate activates the skeletal muscle ryanodine receptor. *Biochem. J.* 2002; 367:423–431. [PubMed: 12102654]
- Jain P, Slama JT, Perez-Haddock LA, Walseth TF. Nicotinic Acid adenine dinucleotide phosphate analogues containing substituted nicotinic Acid: Effect of modification on ca(2+) release. *J. Med. Chem.* 2010; 53:7599–7612. [PubMed: 20942470]
- Kiselyov K, Chen J, Rbaibi Y, Oberdick D, Tjon-Kon-Sang S, Shcheynikov N, Muallem S, Soyombo A. TRP-ML1 is a lysosomal monovalent cation channel that undergoes proteolytic cleavage. *J. Biol. Chem.* 2005; 280:43218–43223. [PubMed: 16257972]
- Lamos SM, Krusemark CJ, McGee CJ, Scalf M, Smith LM, Belshaw PJ. Mixed isotope photoaffinity reagents for identification of small-molecule targets by mass spectrometry. *Angew. Chem. Int. Ed. Engl.* 2006; 45:4329–4333. [PubMed: 16733838]
- Langhorst MF, Schwarzmann N, Guse AH. Ca²⁺ release via ryanodine receptors and Ca²⁺ entry: Major mechanisms in NAADP-mediated Ca²⁺ signaling in T-lymphocytes. *Cell Signal.* 2004; 16:1283–1289. [PubMed: 15337527]
- Lee HC. Nicotinic acid adenine dinucleotide phosphate (NAADP)-mediated calcium signaling. *J. Biol. Chem.* 2005; 280:33693–33696. [PubMed: 16076847]
- Lee HC. Cyclic ADP-ribose and NAADP: Fraternal twin messengers for calcium signaling. *Sci. China Life Sci.* 2011; 54:699–711. [PubMed: 21786193]
- Lee HC, Aarhus R. A derivative of NADP mobilizes calcium stores insensitive to inositol trisphosphate and cyclic ADP-ribose. *J. Biol. Chem.* 1995; 270:2152–2157. [PubMed: 7836444]
- Lee HC, Aarhus R. Functional visualization of the separate but interacting calcium stores sensitive to NAADP and cyclic ADP-ribose. *J. Cell Sci.* 2000; 113(Pt 24):4413–4420. [PubMed: 11082034]
- Lin-Moshier Y, Walseth TF, Churamani D, Davidson SM, Slama JT, Hooper R, Brailoiu E, Patel S, Marchant JS. Photoaffinity labeling of nicotinic acid adenine dinucleotide phosphate (NAADP) targets in mammalian cells. *J. Biol. Chem.* 2012; 287:2296–2307. [PubMed: 22117075]
- Mojzisova A, Krizanovova O, Zacikova L, Kominkova V, Ondrias K. Effect of nicotinic acid adenine dinucleotide phosphate on ryanodine calcium release channel in heart. *Pflugers Arch.* 2001; 441:674–677. [PubMed: 11294249]
- Ogunbayo OA, Zhu Y, Rossi D, Sorrentino V, Ma J, Zhu MX, Evans AM. Cyclic adenosine diphosphate ribose activates ryanodine receptors, whereas NAADP activates two-pore domain channels. *J. Biol. Chem.* 2011; 286:9136–9140. [PubMed: 21216967]
- Pereira GJS, Hirata H, Fimia GM, do Carmo LCG, Bincoletto C, Han SW, Stilhano RS, Ureshino RP, Bloor-Young D, Churchill G, Piacentini M, Patel S, Smaili SS. Nicotinic acid adenine dinucleotide phosphate (NAADP) regulates autophagy in cultured astrocytes. *J. Biol. Chem.* 2011; 286:27875–27881. [PubMed: 21610076]
- Robinette D, Neamati N, Tomer KB, Borchers CH. Photoaffinity labeling combined with mass spectrometric approaches as a tool for structural proteomics. *Expert. Rev. Proteomics.* 2006; 3:399–408. [PubMed: 16901199]
- Schweitzer K, Mayr GW, Guse AH. Assay for ADP-ribosyl cyclase by reverse-phase high-performance liquid chromatography. *Analytical Biochemistry.* 2001; 299:218–226. [PubMed: 11730346]
- Steen M, Kirchberger T, Guse AH. NAADP mobilizes calcium from the endoplasmic reticular Ca(2+) store in T-lymphocytes. *J. Biol. Chem.* 2007; 282:18864–18871. [PubMed: 17446167]
- Thingholm TE, Jensen ON, Larsen MR. Analytical strategies for phosphoproteomics. *Proteomics.* 2009; 9:1451–1468. [PubMed: 19235172]
- Walseth TF, Lin-Moshier Y, Jain P, Ruas M, Parrington J, Galione A, Marchant JS, Slama JT. Photoaffinity labeling of high affinity nicotinic acid adenine dinucleotide phosphate (NAADP)-binding proteins in sea urchin egg. *J. Biol. Chem.* 2012; 287:2308–2315. [PubMed: 22117077]

- Weber PJ, Beck-Sickinger AG. Comparison of the photochemical behavior of four different photoactivatable probes. *J. Pept. Res.* 1997; 49:375–383. [PubMed: 9211218]
- Yamaguchi S, Jha A, Li Q, Soyombo AA, Dickinson GD, Churamani D, Brailoiu E, Patel S, Muallem S. Transient receptor potential mucolipin 1 (TRPML1) and two-pore channels are functionally independent organellar ion channels. *J. Biol. Chem.* 2011; 286:22934–22942. [PubMed: 21540176]
- Zhang F, Jin S, Yi F, Li PL. TRP-ML1 functions as a lysosomal NAADP-sensitive Ca²⁺ release channel in coronary arterial myocytes. *J. Cell Mol. Med.* 2009; 13:3174–3185. [PubMed: 18754814]
- Zhang F, Xu M, Han WQ, Li PL. Reconstitution of lysosomal NAADP-TRP-ML1 signaling pathway and its function in TRP-ML1(–/–) cells. *Am. J. Physiol. Cell Physiol.* 2011; 301:C421–C430. [PubMed: 21613607]
- Zong X, Schieder M, Cuny H, Fenske S, Gruner C, Rotzer K, Griesbeck O, Harz H, Biel M, Wahl-Schott C. The two-pore channel TPCN2 mediates NAADP-dependent Ca(2+)-release from lysosomal stores. *Pflugers Arch.* 2009; 458:891–899. [PubMed: 19557428]

Figure 1. Photoaffinity labeling of Jurkat T cell S100 protein fraction with $[^{32}\text{P}]\text{-}5\text{N}_3\text{-NAADP}$
 Photolabeling of S100 was performed in the absence or presence of the indicated concentrations of various competitors. **(Panel A)** Phosphor image of a gel examining the effect of increasing concentrations of NAADP, and NADP. **(Panel B)** Phosphor image of a gel examining the effect of nicotinic acid (NA), nicotinamide (nic), NAAD, NAD, and NMN. The lane labeled CON is a control. **(Panel C)** Densitometric analyses of experiments similar to those shown in Figure 1(A) with concentrations of NAADP and NADP up to 10 μM .

Figure 2. Photoaffinity labeling of Jurkat T cell P100 protein fraction with [32 P]-5N $_3$ -NAADP
 Photolabeling of Jurkat P100 was performed in the absence or presence of the indicated concentrations of various competitors. **(Panel A)** phosphor image of a gel examining the effect of increasing concentrations of NAADP, and NADP. **(Panel B)** Densitometric analyses of experiments similar to those shown in Figure 2(A).

Figure 3. Characterization of photoaffinity labeled S100 cytosolic NAADP BPs by 2-dimensional IEF/SDS-PAGE

Jurkat S100 was photoaffinity labeled in the absence (**panel A**) or presence (**panel B**) of 500 nM unlabeled NAADP. The samples were then subjected to 2-dimensional gel electrophoresis. Photolabeled proteins that decrease when labeled in the presence of 500 nM NAADP are circled in red. Photolabeled proteins that increase when labeled in the presence of 500 nM NAADP are circled in green. Phosphor images of the dried gels are shown.

Figure 4. Characterization of photoaffinity labeled P100 membrane fraction NAADP BPs by 2-dimensional IEF/SDS-PAGE

Jurkat P100 was photoaffinity labeled in the absence (**panel A**) or presence (**panel B**) of 500 nM unlabeled NAADP. The samples were then subjected to 2-dimensional gel electrophoresis. Photolabeled proteins that decrease when labeled in the presence of 500 nM NAADP are circled in red. Photolabeled proteins that increase when labeled in the presence of 500 nM NAADP are circled in green. Phosphor images of the dried gels are shown.