Nicotinic acid adenine dinucleotide phosphate (NAADP) is the most potent activator of Ca\(^{2+}\) release from intracellular stores known today. Although recent reports have suggested an important function of NAADP in human T lymphocytes, direct evidence for receptor-induced formation of NAADP is yet missing in these cells. Thus, we developed a highly specific and sensitive enzyme assay capable of quantifying low fmol amounts of NAADP. In unstimulated T cells, the NAADP concentration amounted to 4.4 ± 1.6 nM (0.055 ± 0.028 pmol/mg of protein). Stimulation of the cells via the T cell receptor/CD3 complex resulted in biphasic elevation kinetics of cellular NAADP levels and was characterized by a bell-shaped concentration-response curve for NAADP. In contrast, the NAADP concentration was elevated neither upon activation of the ADP-ribose/TRPM2 channel Ca\(^{2+}\) signaling system nor by an increase of the intracellular Ca\(^{2+}\) concentration upon thapsigargin stimulation. T cell receptor/CD3 complex-mediated NAADP formation was dependent on the activity of tyrosine kinases because genistein completely blocked NAADP elevation. Thus, we propose a regulated formation of NAADP upon specific stimulation of the T cell receptor/CD3 complex, suggesting a function of NAADP as a Ca\(^{2+}\)-mobilizing second messenger during T cell activation.

During a rise of the intracellular Ca\(^{2+}\) concentration, Ca\(^{2+}\) either enters the cell from the extracellular space or is released from intracellular stores. The latter process is regulated by an expanding group of intracellular messengers (1, 2) including d-\(\alpha\)-myo-inositol 1,4,5-trisphosphate (InsP\(_3\))\(^3\), cyclic ADP-ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP). In addition, Ca\(^{2+}\) itself co-modulates Ca\(^{2+}\) release via both InsP\(_3\) receptors and ryanodine receptors (RyR).

Clapper et al. (3) reported a Ca\(^{2+}\) releasing activity of a contaminating in commercially available NADP preparations. Nearly a decade later, this contamination was identified as NAADP, which turned out to be the most potent Ca\(^{2+}\)-mobilizing compound in sea urchin egg homogenates (4). NAADP is active in a variety of cells, ranging from plants to animals (for reviews, see Refs. 5–7). Interestingly the functional properties of NAADP-induced Ca\(^{2+}\) release in sea urchin egg homogenates are different from the systems activated by InsP\(_3\) or cADPR (4). Moreover because extracellular stimulation induces increases in intracellular NAADP levels, a second messenger function for NAADP has been proposed in a few experimental systems (for reviews, see Refs. 8 and 9), including murine pancreatic beta cells (10), murine pancreatic acinar cells (11), and arterial smooth muscle cells (12). However, the determination of NAADP is still difficult because the data published so far were obtained by a radioligand binding assay requiring sea urchin egg homogenates as well as \(^{32}\)P-labeled NAADP (10–14).

The molecular identity of the NAADP receptor is still controversial. Although a specific receptor has been proposed in sea urchin eggs (4, 15, 16), arterial smooth muscle cells (17), and microsomes from brain (18) or heart (19), a direct activation of RyR by NAADP has been reported upon reconstitution in artificial membranes (20, 21). Furthermore NAADP-induced Ca\(^{2+}\) release via RyR has been described for MIN6 cells (22), pancreatic acinar cells (23, 24), and human T cells (25, 26). Galione and co-workers (10, 12, 27, 28) recently reported the release of Ca\(^{2+}\) by NAADP from a lysosomal compartment. Thus, both a "one pool-one receptor" mechanism (NAADP directly activates RyR) and a "two-pool" mechanism (NAADP releases Ca\(^{2+}\) from a lysosomal store, which than induces Ca\(^{2+}\) release by RyR) may be used by different cell types (for a review, see Ref. 29). In T lymphocytes an important function of NAADP in the process of Ca\(^{2+}\) signaling induced by stimulation of the T cell receptor (TCR)/CD3 complex has been reported (30). Thus, an easy, sensitive, and reliable method to quantify cellular NAADP concentrations would be highly important to analyze a second messenger function in this cell type.

In the present study, we describe an improved enzymatic cycling assay for the accurate determination of NAADP, allowing the quantification of low fmol amounts. All enzymes used for this assay are commercially available, and neither sea urchin egg homogenates nor radioactively labeled NAADP are required. This method was used for the determination of cellular NAADP concentrations in T cells. Upon stimulation of the cells via the TCR/CD3 complex, we found a biphasic elevation of NAADP levels. A bell-shaped concentration-response curve was obtained upon stimulation of the TCR/CD3 complex. In contrast, no NAADP elevation was detectable upon stimulation of the ADP-ribose (ADPR)/TRPM2 Ca\(^{2+}\) signaling pathway (31) or upon an increase of [Ca\(^{2+}\)]\(_{\text{cyt}}\), induced by thapsigargin. Therefore, we propose a second messenger function of NAADP in T cells.
Experimental Procedures

Materials—Resazurin (R2127), ADP-ribosyl cyclase (A8950), diaphorase (D5540), glucose-6-phosphate dehydrogenase (G8164), NAD glycohydrolase (N9629), nucleotides, and other standard compounds were purchased from Sigma (catalog numbers are in parentheses) or from Roche Applied Science. Trichloroacetic acid, diethyl ether, and trifluoroacetic acid were obtained from Merck, and Q-Sepharose Fast Flow was from GE Healthcare. Charcoal Norit A, obtained from Serva (Heidelberg, Germany), was activated by treatment with 1 M HCl by boiling for 30 min and washed with water until the pH value was neutral. PP2 and genistein were from Calbiochem. All other chemicals were of the highest purity available. SeralPur water (Seral, Ransbach, Germany) was used during all experiments.

Cell Culture—Jurkat T cells (subclone JMP) were cultured as described previously (32). Briefly the cells were kept in RPMI 1640 medium with 25 mM HEPES, pH 7.4, and Glutamax I supplemented with 100 units/ml penicillin, 50 μg/ml streptomycin, and 7.5% (v/v) newborn calf serum (Biochrom, Berlin, Germany) at a density of 0.3–1.0 × 10^6 cells/ml. Counting of the cells and determination of the cell volume were performed using a CASY TT 1 system (Schärfe System, Reutlingen, Germany). To quantify cellular protein content, the cells were lysed in 25 mM HEPES, pH 7.2, in the presence of Complete protease inhibitor mixture (Roche Applied Science) by incubation for 10 min on ice followed by ultrasonic disruption for 30 s. Thereafter, the protein concentration was determined with Bradford reagent (Bio-Rad) using BSA as standard.

Extraction of Cellular NAADP—1.6 × 10^8 Jurkat cells were harvested by centrifugation (500 × g for 5 min at room temperature), resuspended in 16 ml of buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgSO_4_, 1 mM CaCl_2_, 1 mM NaH_2PO_4_, 5.5 mM glucose, and 20 mM HEPES, pH 7.4, and kept at room temperature for 40 min. After this incubation, the cell suspension was divided into two identical halves (“twin samples”), and the volume was adjusted to 10 ml with the same buffer as above. The cells in both parts of the twin samples were then stimulated for different periods of time with either different concentrations of OKT3, a vehicle control, 100 μg/ml concanavalin A, or 100 mM thapsigargin. In some experiments, 1 μM PP2 was preincubated with cells for 5 min, or 80 μg/ml genistein was preincubated with cells for 10 min; then the cells were stimulated for 5 min with 5 μg/ml OKT3. After rapid centrifugation (960 × g for 1 min at 10 °C), the cell pellets were resuspended in 1 ml of ice-cold trichloroacetic acid solution (20%, w/v) and lysed using a Potter-Elvehjem homogenizer (five strokes at 1500 rpm). Thereafter the samples were frozen in liquid nitrogen and thawed at 37 °C two times, cell debris and precipitated proteins were removed by centrifugation (4400 × g for 10 min), and the supernatants were collected. One of each of a pair of twin samples was spiked by addition of 15 pmol of NAADP to calculate the recovery of the extraction procedure. Then the samples were repeatedly extracted with 5 volumes of water-saturated diethyl ether until the pH value was nearly neutral and freeze-dried for 60 min to remove traces of ether. In some experiments artificial samples were used consisting of 0.5 ml of BSA solution (5 mg/ml in water) and 15 pmol of NAADP and/or 37.5 pmol of purified NADase (see below). These samples were processed in the same way as described for the cell extract samples.

Purification of Cell Extracts by Gravity-fed Anion-exchange Chromatography—For the anion-exchange chromatography of neutralized trichloroacetic acid extracts, plastic filtration tubes (Supleco, Bellefonte, PA) were packed with 500 μl of Q-Sepharose Fast Flow, cleaned in place with 5 ml of 150 mM trifluoroacetic acid, and equilibrated with 15 ml of 10 mM Tris/HCl, pH 8.0. The cell extracts were diluted with 10 ml of 10 mM Tris/HCl, pH 8.0, and applied to the columns in aliquots of 1 ml. The columns were then washed with 10 ml of 10 mM Tris/HCl, pH 8.0. For the elution of NAADP, trifluoroacetic acid solutions of increasing concentrations (each 1.5 ml of 10, 12.5, 15, 17.5, 20, and 25 mM) were used. The eluate fractions were rapidly diluted with 3.5 ml of ice-cold water and freeze-dried. These samples were stored at −80 °C and analyzed as soon as possible. To determine the elution profile of NAADP, artificial samples consisting of BSA with or without 15 pmol of NAADP were extracted as detailed above. In this case, all eluate fractions were separately cleaved with NAD glycohydrolase (NADase) and then analyzed by the cycling assay (see below). As standard procedure the 17.5–25 mM trifluoroacetic acid fractions containing the NAADP were pooled and further processed.

Removal of Interfering Compounds by Cleavage with NAD Glycohydrolase—NAADP standards or other compounds were dissolved in 1.5 ml of buffer containing 1 mM Tris/HCl and 2.5 mM MgCl_2_, pH 7.3. For the determination of cellular NAADP amounts, the 17.5–25 mM pool of trifluoroacetic acid fractions was used. This sample was reconstituted in 1.5 ml of the same buffer as above. To remove interfering nucleotides, 0.1 unit of the purified NADase was added to the samples. After incubation for 4 h at 37 °C, the enzyme was inactivated for 20 min at 95 °C, and the samples were cooled on ice and then directly used for the cycling assay.

Purification of NAADP—NAADP was purified by gravity-fed anion-exchange chromatography because commercially available NADP preparations always contain a contamination of NAADP. About 10 μmol of NAADP (Roche Applied Science) were dissolved in 20 ml of 1 mM Tris/HCl, pH 8.0, and applied to a column of 1 ml of Q-Sepharose Fast Flow that was prepared as detailed above. NAADP was then eluted using increasing concentrations of trifluoroacetic acid (4 ml of each 2, 4, 6, 8, 10, 12, and 15 mM). The NAADP concentration in each fraction was determined by measuring the UV absorption at 260 nm (and initially also by HPLC). After freeze-drying, the purified NAADP, which eluted mainly in the 10 mM trifluoroacetic acid fraction, was dissolved at a concentration of 10 mM, and the purity was additionally checked by HPLC.

Purification of Enzymes—To purify commercially available NADase, 2.9 mg (1.6 units) of the lyophilized enzyme was dissolved in 320 μl of buffer containing 1 mM Tris/HCl and 2.5 mM MgCl_2_, pH 7.0; mixed with 320 μl of BSA solution (5 mg/ml in water) and 640 μl of a suspension (2%, w/v) of activated charcoal in 150 mM NaCl and 20 mM NaH_2PO_4_, pH 8.0 adjusted with NaOH; and incubated for 30 min at 37 °C with vigorous shaking. The charcoal was removed by centrifugation at 11,000 × g for 10 min at 4 °C, and the supernatant was transferred to a Centricon YM-10 device (cutoff, 10 KDa; Millipore). The enzyme solution was concentrated (4400 × g for 20 min at 4 °C) to a volume of 150 μl and stored at −20 °C.

Glucose-6-phosphate dehydrogenase and diaphorase were purified as follows. 100 μl of each enzyme solution (1 mg/ml glucose-6-phosphate dehydrogenase and 12 mg/ml diaphorase, both in 50 mM NaH_2PO_4_, pH 8.0 adjusted with NaOH) were mixed with 200 μl of BSA solution (5 mg/ml in water) and 1.2 ml of a suspension (2%, w/v) of activated charcoal in 50 mM NaCl and 20 mM NaH_2PO_4_, pH 8.0 adjusted with NaOH. After incubation at 37 °C for 30 min, the charcoal was removed by centrifugation (11,000 × g for 10 min at 4 °C), and the supernatant was used directly for the cycling assay.

Cycling Assay for NAADP—In the first reaction of the enzymatic cycling assay, NAADP was converted into NADP (see Fig. 1A). Because all measurements were done in triplicate, six wells of a microplate were filled with each 200 μl of the samples, which had been incubated with...
the NADase before, or with standard NAADP in the same buffer. In some experiments, standard NAADP was incubated with 10 units/ml alkaline phosphatase in the same buffer at 37 °C overnight, and the enzyme was subsequently removed using a Centricron YM-10 device (4400 × g for 20 min at 4 °C). To three of the wells, 11 μl of a solution containing 0.55 mM nicotinamide, 36 mM sodium acetate, pH 4.2, and 9.1 μg/ml ADP-ribosyl cyclase (ADPRC) were added. 11 μl of the same solution containing BSA instead of the enzyme were added to each of the three remaining wells. The microplate was sealed with Parafilm and incubated for 60 min in the dark at room temperature. Then the amplification and indicator reaction (see Fig. 1A) were started by addition of 59 μl of cycling mixture to each well. The cycling mixture contained (all solutions in water except the purified enzymes) 1 μl of 1 mM nicotinamide, 20 μl of 500 mM NaH2PO4, pH 8.0 adjusted with NaOH, 1 μl of 1 mM flavin mononucleotide, 2 μl of 5 mg/ml BSA, 0.1 μl of 10 mM resazurin, 5 μl of 100 mM glucose-6-phosphate, and 30 μl of the purified enzymes (see above). Directly after addition of the cycling mixture, the fluorescence (excitation, 544 nm; emission, 590 nm) was measured for each well by a microplate reader (Victor 4120, Wallac, Freiburg, Germany). The microplate was then sealed with Parafilm and incubated for 12 h in the dark at room temperature. The fluorescence was measured again, and the differences between end and start of the reaction were determined for each well. To correct the obtained values for the background due to the presence of NAADP or unspecific oxidation of resazurin, each rise in fluorescence in the absence of the enzyme (see Fig. 1B) was corrected for the fluorescence increase in the absence of the enzyme (see Fig. 1B). The NAADP content in the samples that were purified by anion-exchange chromatography was obtained by addition of the pooled 17.5–25 mM trifluoroacetic acid fraction. For each pair of twin samples, the recovery was determined by calculating the difference between the halves of the sample with and without spike. Finally the NAADP amount in the twin sample without exogenous NAADP was corrected for the respective recovery.

Measurement of [Ca2+]i.—Loading of Jurkat T cells with Fura2/AM (Molecular Probes) and determination of [Ca2+]i, was performed as described previously (32, 33). The cells were stimulated with different concentrations of OKT3 or a vehicle control. For the quantification of the increase in [Ca2+]i, (see Fig. 3), both the peak and the plateau (500 s after stimulation) were corrected for the basal Ca2+ concentration.

Statistical Analysis.—For determination of statistical significance, data were tested using a one-way analysis of variance in combination with a Student-Newman-Keuls test. Statistical significance was considered for p < 0.05.

RESULTS

The principle of the cycling assay for NAADP is depicted in Fig. 1A. The assay consists of three enzymatic reactions. In the first reaction (Fig. 1A, upper panel), NAADP is converted into NADP using the ADPRC from Aplysia californica. This reaction was conducted at an acidic pH of 4.2 and in the presence of a high excess of nicotinamide, thus reversing the enzymatic formation of NAADP from NADP by ADPRCs (34). The NAADP produced by the ADPRC is then cycled by two enzymatic reactions (Fig. 1A, lower right panel, amplification). In the first reaction, glucose-6-phosphate dehydrogenase from Saccharomyces cerevisiae reduces NADP to NADPH, which is then reoxidized to NADP by diaphorase from Clostridium kluyveri. Simultaneously to the reoxidation of NADPH, diaphorase catalyzes the conversion of resazurin into the highly fluorescent resorufin (Fig. 1A, lower left panel, indicator reaction). The major advantage of this kind of cyclic enzyme assay is the strong amplification of the initial signal because the presence of one
NADP molecule results in the formation of many molecules of resoru-
fin, thus allowing the detection of minute amounts of NADP.

The first step to establish the assay described above was to adopt the
cycling reaction for NADP from the literature (35, 36) and to optimize it.
For this purpose, the cycling reaction was performed in the presence of
NADP without prior conversion of NAADP into NADP (data not
shown). A very crucial point was the quality of the enzymes used in
the cycling reaction: because the enzymes commercially available were not
sufficiently pure (which resulted in a high fluorescence background
even in the absence of any substrate in the cycling reaction), both
enzymes were purified using activated charcoal in the presence of BSA
(see “Experimental Procedures”). After this purification, only a very
small increase in the resorufin fluorescence was detected in the absence
of NADP (data not shown).

The complete conversion of NAADP to NADP by the ADPRC is
essential to establish an assay that indirectly quantifies NAADP after
conversion into NADP. The ADPRC from *A. californica* catalyzes the
synthesis of NADP from NAADP at an acidic pH and in the presence of
high concentrations of nicotinic acid (34). Different conditions to
reverse this reaction were tested and analyzed by HPLC separation of
the reaction products (data not shown). At an acidic pH value, the
ADPRC converted NAADP into NADP at room temperature, whereas
no reaction was observed at 10 °C. Besides NAADP, which was the main
product of the reaction, also small amounts of 2’-phospho-ADP-ribose
were generated. Next the formation of NADP from NAADP was cou-
pled to the enzymatic cycling reaction. The reaction conditions of the
NAADP formation from NADP were extensively optimized, i.e. differ-
ent buffers, pH values, concentrations of nicotinamide, amounts of
ADPRC, temperatures, and incubation times were tested. The optimal
condition to convert NAADP into NADP was an incubation for 60 min
at 23 °C in the presence of 29 mM nicotinamide, 1.88 mM sodium ace-
tate, pH 4.2, and 0.474 μg/ml ADPRC. After this optimization was com-
pleted, a calibration of the cycling assay was performed using different
amounts of NAADP standards in the range from 25 to 1000 fmol (data
not shown). A linear correlation between NAADP amount and fluores-
cence increase after 12 h was found (*r* = 0.9996), and the detection
limit was 25 fmol at a signal/noise ratio of >3.

Another important point to consider is the presence of NADP in cell
extracts because NADP would also be detected by the cycling reaction,
resulting in a false positive signal. To remove endogenous NADP, both
gravity-fed anion-exchange chromatography and an enzymatic digest
using an NADase from *Neurospora crassa* were used. A large portion
of NADP was eluted at 10 mm trifluoroacetic acid, whereas NAADP eluted
in fractions 17.5–25 mM (Fig. 1E). The remaining contamination of
NADP was removed by NADase digest. This enzyme hydrolyzes NAD
to ADPR and nicotinamide but is also capable of cleaving NADP into
2’-phospho-ADP-ribose and nicotinamide. In contrast, NAADP is
resistant to NADase treatment (37). Because it was not known whether
the enzymatic removal of NADP using the NADase would be complete,
we used the following procedure to correct for a possible background
of NADP. After the cleavage using the NADase, all samples were incuba-
ted either with or without ADPRC in the first reaction of the assay.
Then the enzymatic cycling was performed with both types of samples,
and the fluorescence increase was quantified after 12 h. Because
NAADP cannot be converted into NADP in the absence of the ADPRC,
the NADP background was quantified by the reactions without ADPRC.
Thus, the fluorescence increase in the presence of ADPRC was cor-
rected for the increase in the absence of the enzyme for each sample.

The use of the NADase resulted in a decrease in the sensitivity of the
assay. This may be due to a co-precipitation of NAADP with the dena-
tured enzyme during the heat inactivation of the NADase. However,
the correlation between the amount of NAADP and the fluorescence
increase was still linear as depicted in Fig. 1C. Under these conditions,
the detection limit for NAADP was 50 fmol at a signal/noise ratio of >3.
Obviously the correction of the fluorescence difference in the presence
of ADPRC (Fig. 1B, *black bars*) for the fluorescence increase in the
absence of the enzyme (Fig. 1B, *light gray bars*) resulted in a complete
correction of the background (see blank value in Fig. 1B). Possibly this
background in the absence of NADP was due to partial spontaneous
oxidation of resazurin.

To investigate the specificity of the assay, NAADP was incubated
with alkaline phosphatase, leading to the degradation of NAADP to
NAAD (Fig. 1D) (37). In this case, no fluorescence increase was detect-
able, demonstrating the specificity of the assay.

For the determination of cellular NAADP concentrations, the cycling
assay has to be combined with a method for the extraction and purifi-
cation of cellular nucleotides (see below). This procedure takes advan-
tage of anion-exchange chromatography using Q-Sepharose and elu-
tion with increasing concentrations of trifluoroacetic acid. To obtain
the elution profile of NAADP, an artificial sample containing BSA and
NAADP was extracted and purified by anion-exchange chromatogra-
phy, and the NAADP amount in each fraction was quantified separately
(Fig. 1F). In these experiments, the main amount of NAADP was found
in the trifluoroacetic acid fractions 17.5 and 20 mM, thus as a standard
procedure the 17.5–25 mM trifluoroacetic acid fractions were pooled.
To exclude a degradation of NADP into NAADP during the acidic
extraction procedure, we used artificial samples consisting of BSA and
NAADP or NAADP (Fig. 1F). For these experiments, the NAADP standard
was purified prior to use to make sure that no contamination of NAADP
was present. Purified NAADP showed no increase in the fluorescence
difference (Fig. 1F). Moreover no increase in the NAADP signal in a
mixed sample consisting of NAADP and excess NADP was found (Fig.
1F, *rightmost bar*), ruling out an artificial formation of NAADP from
NADP.

Next 19 compounds that are likely present in cell extracts were tested
for an interference with the NAADP cycling assay (Table 1). A 1000-fold

### TABLE 1

**Endogenous nucleotides do not interfere with the cycling assay**

| Compound                          | ΔFluorescence (mean ± S.D.) |
|-----------------------------------|-----------------------------|
| NAADP (control)                   | 100 ± 3.7                   |
| NAD                              | 105.6 ± 1.5                 |
| ATP                              | 100.3 ± 1.1                 |
| ADP                              | 111.3 ± 2.4                 |
| AMP                              | 111.8 ± 1.4                 |
| cADPR                            | 97.4 ± 1.9                  |
| NAAD                             | 99.5 ± 2.6                  |
| ADPR                             | 106.7 ± 2.5                 |
| GTP                              | 95.8 ± 2.2                  |
| Nicotinamide                      | 97.4 ± 1.9                  |
| Nicotinic acid                    | 97.2 ± 2.1                  |
| NADP                             | 97.1 ± 2.0                  |
| 2’-Phospho-cADPR                  | 100.4 ± 3.4                 |
| 2’-Phospho-ADPR                   | 94.6 ± 2.3                  |
| Nicotinic amide mononucleotide    | 103.5 ± 2.4                 |
| cAMP                             | 101.8 ± 2.6                 |
| cGMP                             | 106.4 ± 0.8                 |
| IMP                              | 95.6 ± 0.7                  |
| IDP                              | 102.5 ± 2.0                 |
| GDP                              | 98.1 ± 1.5                  |

Endogenous nucleotides do not interfere with the cycling assay.

Cellular nucleotides and related compounds were tested for an interference with the
NAADP cycling assay. 500 fmol of NAADP were quantified either without any
addition (control) or in the presence of a 1000-fold excess of the compounds indi-
cated. The samples were digested using NADase from *N. crassa* and then analyzed
by the enzymatic cycling assay. Given are the differences in fluorescence between
the reactions with and without ADPRC in the first reaction of the assay normalized
to the control (n = 3, mean ± S.D.).

### References

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The objective of the NAADP assay described in this study was the quantification of cellular NAADP amounts. To allow this, a protocol for the extraction of endogenous NAADP was developed that was compatible with the enzymatic NAADP assay. In this extraction procedure an acidic lysis of the cells was used that resulted in an immediate denaturation of cellular enzymes, thus inactivating any possible cellular NAADP metabolism. This is an important point for the determination of cellular messengers because their concentration must not change during the extraction. For this reason, the twin samples of Jurkat T cells were lysed in trichloroacetic acid in a Potter-Elvehjem homogenizer. After removal of precipitated proteins and cell debris, one of the twin samples was spiked using a known amount of exogenous NAADP to calculate the recovery of the extraction procedure. Trichloroacetic acid was removed by ether extraction, and the samples were purified by anion-exchange chromatography using a strong anion-exchange matrix (Q-Sepharose). Then the NADase digest and the cycling assay were performed as described above. By comparison of the difference between the two twin samples and the amount of NAADP used for spiking, the recovery was determined for each sample. Then the NAADP amount determined in the non-spiked twin sample was corrected for the respective recovery.

Using this procedure, basal NAADP concentrations of 4.4 ± 1.5 nM (n = 7, mean ± S.D.) were determined in Jurkat T cells (Fig. 2A) assuming an even distribution of NAADP inside the cells. The cell volume was determined for each preparation of cells to calculate the intracellular NAADP concentration. The mean cellular volume amounted to 1.32 ± 0.33 µl/10⁶ cells (n = 28, mean ± S.D.). When normalized to cellular protein levels, the basal NAADP concentration was 0.055 ± 0.028 pmol/mg of protein. The mean recovery for NAADP was 43.5 ± 18% (n = 7, mean ± S.D.). Upon stimulation of the cells with 5 µg/ml OKT3, we found an instantaneous elevation of the NAADP concentration up to 33.6 ± 7.2 nM (n = 4, mean ± S.D.), corresponding to 0.245 ± 0.056 pmol/mg of protein (Fig. 2, A and C). This increase was transient because 30 s after the stimulation it was reduced almost to the basal level (Fig. 2, A and C). However, the NAADP elevation was biphasic because a second elevation was detectable 5 min after stimulation (Fig. 2A) that was long lasting (up to 20 min after stimulation). Afterwards the NAADP concentration decreased again to the basal level. Comparison of the kinetics of NAADP formation with global changes in [Ca²⁺]i, using the same stimulus demonstrated that NAADP appears to act in the initial, so-called pacemaker phase of T cell Ca²⁺ signaling (Fig. 2C). In addition, also in the Ca²⁺ signaling plateau phase between 5 and 20 min, NAADP is likely to be involved (Fig. 2, A and B).

Next we investigated the concentration-response relationship between NAADP elevation and TCR/CD3 stimulation (Fig. 3). In these experiments the cells were stimulated for a constant period of time (5 min) with different concentrations of OKT3 ranging from 0.01 to 10 µg/ml. Even at 0.01 µg/ml a significant increase in NAADP levels was found, whereas the most effective OKT3 concentration was 0.1 µg/ml. At higher OKT3 concentrations, the NAADP levels were still elevated as compared with the control but decreased slightly. Interestingly stimulation with a high concentration of OKT3 (10 µg/ml) did not significantly increase the basal NAADP concentration. To directly compare the NAADP elevation to global Ca²⁺ mobilization, we stimulated Jurkat cells with different concentrations of OKT3 and determined the changes in [Ca²⁺]i, for both the peak (Fig. 3B) and the plateau phase (Fig. 3C) of the Ca²⁺ signal. In these experiments, stimulation with higher concentrations of OKT3 resulted in higher Ca²⁺ signals with an EC₅₀ of about 0.1 µg/ml. Comparison of these concentration-response curves demonstrate that OKT3 concentrations submaximal for increases in global [Ca²⁺], are optimal for NAADP formation (see “Discussion”).

To further characterize the signal transduction pathways leading to NAADP formation upon TCR/CD3 stimulation, we treated Jurkat T cells with OKT3 in the presence of the Tyr kinase inhibitors PP2 and genistein (Fig. 4). Inhibition of the Tyr kinases p56lck and p59fyn by PP2...
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had no significant effect on NAADP formation upon OKT3 stimulation, whereas a more broad inhibition of Tyr kinases using genistein completely blocked the OKT3-induced NAADP elevation (Fig. 4). Similarly determination of global Ca^{2+} signaling in response to OKT3 in the presence of genistein resulted in a significant decrease of both the peak and plateau Ca^{2+} signaling phase (Δ peak: OKT3/vehicle, 435 ± 62 nm; OKT3/genestein, 188 ± 55 nm, p = 0.001; Δ plateau: OKT3/vehicle, 225 ± 17 nm; OKT3/genestein, 60 ± 23 nm, p = 0.0001).

Furthermore stimulation of Jurkat cells with high concentrations of the lectin concanavalin A, a condition known to induce Ca^{2+} entry and apoptosis via the ADPR/TRPM2/Ca^{2+} signaling pathway in T lymphocytes (31, 39–41), did not significantly increase cellular NAADP concentrations (Fig. 4). Finally a pharmacologically induced rise of [Ca^{2+}], by treatment of the cells with the sarco(endo)plasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin did not significantly elevate cellular NAADP levels (Fig. 4).

**DISCUSSION**

The enzymatic cycling assay described in the present study is capable of detecting low fmol amounts of NAADP, and no interference of 19 nucleotides or related endogenous compounds was found. Contaminating NADP was efficiently removed by anion-exchange chromatography and NADase treatment, allowing an accurate determination of NAADP. Because our assay showed a high sensitivity combined with a remarkable specificity, the accurate determination of cellular NAADP concentrations in human T lymphocytes was possible. Stimulation of Jurkat T cells via the TCR/CD3 complex resulted in an elevation of cellular NAADP levels. Interestingly a very rapid increase of the NAADP concentration was followed by a reduction to basal levels and a second, somewhat lower but long lasting elevation. The concentration-response curve for NAADP formation upon OKT3 stimulation was bell-shaped with a most effective OKT3 concentration of 0.1 μg/ml. NAADP formation was completely blocked by inhibition of Tyr kinases with genistein, whereas more specific inhibition of p56^{fyn} and p59^{fyn} by PP2 was without effect. Furthermore treatment of T cells with the lectin concanavalin A or the sarco(endo)plasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin had no significant effect on the NAADP concentration.

Enzymatic cycling assays have been used for the determination of various biomolecules, e.g. cGMP (42), NAD (43), and NADP (35, 36). Graeff and Lee (37, 44) significantly improved these assays by using the reduction of resazurin to the highly fluorescent resorufin. In the present study this system was adopted for the determination of NADP, which is generated from NAADP, yielding a detection limit of 25 fmol of NADP using standard compounds without NADase digest. A crucial point for the NAADP determination using a detection system for NADP is the conversion of NAADP to NAD. Although the capability of both A. californica ADPRC as well as human CD38 to produce NAADP from NADP by base exchange is known (34), previously it was unclear whether this enzymatic reaction might be reversible under certain conditions. Because in the present study these conditions were established, it was possible to use this reversed enzymatic reaction in conjunction with our improved cycling assay for NADP.

Graeff and Lee quantified both cADPR (44) and NAADP (37) by enzymatic cycling assays with NAD and NADH as substrates in the amplification reaction. In their NAADP assay, the compound was first dephosphorylated at the 2'-position by treatment with alkaline phosphatase (37). Then the resulting NAAD was converted into NAD using nicotinamide-mononucleotide adenyltransferase in the presence of nicotinamide mononucleotide (37). Overall this procedure resulted in a detection limit of 300 fmol of NAADP (37). Compared with the method

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**FIGURE 3.** Concentration-response curve of NAADP formation upon TCR/CD3 stimulation. A. Jurkat T lymphocytes were stimulated for 5 min with different concentrations of OKT3 or a vehicle control. Endogenous NAADP was extracted and quantified using the cycling assay (n = 2–7, mean ± S.E.; *, p < 0.05 versus the basal value). B, the difference between the peak and the basal concentration in [Ca^{2+}], was determined upon stimulation of the cells with different concentrations of OKT3 (n = 3–6, mean ± S.E.). C, the difference between the plateau 500 s post-stimulation and the basal concentration in [Ca^{2+}], was determined upon stimulation of the cells with different concentrations of OKT3 (n = 3–6, mean ± S.E.).

**FIGURE 4.** Elevation of cellular NAADP levels is specifically induced by TCR/CD3 stimulation and can be blocked by inhibition of Tyr kinases. Jurkat T cells were left untreated (control) or stimulated for 5 min with either OKT3 (5 μg/ml), concanavalin A (ConA, 100 μg/ml), or thapsigargin (Tg, 100 nM). In some experiments, cells were preincubated for 5 min with PP2 (1 μM) or for 10 min with genistein (80 μg/ml) before stimulation with OKT3 (n = 2–7, mean ± S.E.; *, p < 0.05 to the control).
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introduced by Graeff and Lee (37), the assay described in the present study shows some advantages. First, all enzymes are commercially available, whereas nicotinamide-mononucleotide adenyltransferase has to be prepared in a rather sophisticated procedure from porcine liver (45). Second, two enzymatic reactions with incubation overnight were necessary to convert NAADP into NAD, whereas in the present study only one reaction converted NAADP into NADP within 1 h. Third, the detection limit in the present study was about 1 order of magnitude lower as compared with the assay of Graeff and Lee (37). However, one advantage of their assay is the possibility to determine nicotinic acid at micromolar concentrations with the same experimental procedure.

A major concern for all assays determining cellular biomolecules is the presence of potentially interfering compounds. For the assay described in the present study especially the absence of NADP was crucial because NAADP would have been detected by the cycling reaction, leading to false positive results. Thus, the NADase from N. crassa was used to remove NADP because NAADP is not cleaved by NADases (37). This was confirmed by our results (see Table 1) because removal of a 1000-fold NADP excess neither decreased the NAADP signal (by cleavage of NAADP) nor increased the NAADP signal (by incomplete removal of NADP). To be on the safe side, for any sample a correction from sea urchin egg homogenates (13, 14) has been used for this purpose. This method takes advantage of the irreversible binding of the fluorescence in the presence of the ADPRC in the first reaction of the assay with the fluorescence in the absence of ADPRC was performed to ensure that even small NADP amounts possibly remaining after the NADase treatment did not interfere with the assay. Although a mixture of NADase and apyrase was used to remove contamiinations in the assay of Graeff and Lee (37), anion-exchange chromatography and cleavage with NADase alone was sufficient in the present study. Because cell extracts were also purified by anion-exchange chromatography, NAADP was enriched with respect to other compounds from the samples, thus making an interference of other cellular nucleotides even more unlikely.

The previously known NAADP cycling assay has not been used for the determination of cellular NAADP concentrations so far (37). However, a competitive radioligand binding assay using the NAADP receptor from sea urchin egg homogenates (13, 14) has been used for this purpose. This method takes advantage of the irreversible binding of NAADP to its yet uncharacterized receptor (46). With this assay, an NAADP concentration of 4 μM has been determined in activated sea urchin sperms (13) as well as an increase in cellular NAADP levels upon stimulation of pancreatic beta cells with glucose (10), upon stimulation of murine pancreatic acinar cells with cholecystokinin (11), and upon stimulation of arterial smooth muscle cells with endothelin-1 (12). In addition, basal cellular concentrations of NAADP in erythrocytes (16 nM), rat hepatocytes (4.5 nM), and Escherichia coli cells (2.5 nM) have been reported (14). The enzymatic cycling assay described in the present study showed some advantages as compared with the radioligand assay. First, homogenates from sea urchin eggs, which are not commercially available, are not required, and second, no 32P-labeled NAADP needs to be synthesized and purified. The extraction procedure used by Churamani et al. (14) also consists of lysis of the cells in trichloroacetic acid followed by ether extraction and strong anion-exchange HPLC, leading to a recovery of 88%. A major concern of a ligand binding assay using cell homogenates may be a potential metabolic activity present in the cell extract, possibly leading to degradation of NAADP. However, this is not the case for the cycling assay described here.

The cycling assay was used to determine cellular NAADP concentrations in human T lymphocytes. Assuming an even distribution of NAADP inside the cell and not taking solid structures into account, a basal cytosolic NAADP concentration of 4.4 ± 1.5 nM was found. This corresponds well to the concentrations of 4.5–16 nM reported for other eukaryotic cells (14).

Upon stimulation of T lymphocytes via the TCR/CD3 complex, a rapid, ~7.6-fold elevation of the basal NAADP concentration was found. This remarkable increase preceded the global elevation of [Ca2+]i, (Fig. 2C), thus suggesting a role for NAADP during the generation of early, subcellular Ca2+ signals upon TCR/CD3 stimulation as has already been proposed (26). A comparable rapid and transient elevation of NAADP levels has been reported for the stimulation of mouse pancreatic acinar cells with cholecystokinin (11). Also in these cells, a role for NAADP in the generation of small, localized Ca2+ signals preceding global Ca2+-induced Ca2+ release has been hypothesized (11). Up to now the pacemaker phase and the initial global phase of T cell Ca2+ signaling have mainly been attributed to InsP3 because upon OKT3 stimulation InsP3 shows a transient time course with a peak around 3 min (Refs. 32 and 47; for a review, see Ref. 48). The data presented here indicate that NAADP is working in close collaboration with InsP3 in the earliest time window of T cell activation to shape the initial local and global Ca2+ response. Although the initial rise in NAADP in T cells is very similar to the one observed in pancreatic acinar cells, in Jurkat T cells we found a second more prolonged elevation of NAADP. This correlates, at least up to 20 min post-stimulation, with the Ca2+ plateau phase induced by OKT3. However, although we have shown previously that the NAADP target in T cells, the RyR (25, 26), is involved in the sustained Ca2+ signaling phase (33), a causal relation between the two events remains to be demonstrated. Moreover determining the extent of cooperation between the established modulator of RyR, cADPR, which has been shown to regulate the sustained Ca2+ signaling phase (49), and NAADP in the time window between 5 and 20 min will be the subject of future studies.

In contrast to the concentration-response curve for NAADP formation in pancreatic acinar cells upon cholecystokinin (11), in T cells a bell-shaped concentration-response curve was found upon stimulation via the TCR/CD3 complex. Unexpectedly the largest NAADP formation was observed at 0.1 μg/ml OKT3, a concentration that induced an approximately half-maximal global Ca2+ response (Fig. 3). Moreover at 10 μg/ml OKT3 the Ca2+ response was still global, but NAADP formation went down close to controls (Fig. 3). At present the biological meaning of this behavior remains unclear. However, it is well known that different concentrations of anti-CD3 antibodies cause different cellular responses in primary T cells, e.g. high anti-CD3 concentration leads to apoptosis, whereas low concentrations promote proliferation or, in the absence of co-stimulation, may also lead to anergy. Thus, it may well be that NAADP as a Ca2+-mobilizing second messenger plays a dominant role in proliferative or anergic T cell responses.

A rise of NAADP was found neither upon pharmacologically induced rise of [Ca2+]i, using thapsigargin nor upon stimulation of T lymphocytes via the ADPR/TRPM2 signaling system (31). Thus, a specific stimulation via the TCR/CD3 complex of the cells is necessary to increase their NAADP levels. However, the enzymes involved in the formation and degradation of NAADP are still unknown. Tyr kinases are involved in the control of NAADP formation because a broad block of these signaling proteins using genistein completely abolished NAADP elevation. Apparently the Tyr kinases p56Lck and p59Fyn are not involved in this regulatory pathway because specific inhibition by PP2 had no effect on NAADP formation.

According to Lee (8) and Rutter (9), most of the criteria for the establishment of NAADP as a second messenger are now fulfilled. First, cellular NAADP concentrations can be determined using either the NAADP cycling assay or the radioligand binding assay, and second, a
stimulation-induced increase has been demonstrated in different cell systems, including T cells.

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