Nicotinic Acid Adenine Dinucleotide Phosphate ($NAADP^+$) Is an Essential Regulator of T-lymphocyte Ca^{2+}-signaling

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Abstract. Microinjection of human Jurkat T-lymphocytes with nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) dose-dependently stimulated intracellular Ca^{2+}-signaling. At a concentration of 10 nM $NAADP^+$ evoked repetitive and long-lasting Ca^{2+}-oscillations of low amplitude, whereas at 50 and 100 nM, a rapid and high initial Ca^{2+}-peak followed by trains of smaller Ca^{2+}-oscillations was observed. Higher concentrations of $NAADP^+$ (1 and 10 μM) gradually reduced the initial Ca^{2+}-peak, and a complete self-inactivation of Ca^{2+}-signals was seen at 100 μM. The effect of $NAADP^+$ was specific as it was not observed with nicotinamide adenine dinucleotide phosphate. Both inositol 1,4,5-trisphosphate– and cyclic adenosine diphosphoribose–mediated Ca^{2+}-signaling were efficiently inhibited by coinjection of a self-inactivating concentration of $NAADP^+$. Most importantly, microinjection of a self-inactivating concentration of $NAADP^+$ completely abolished subsequent stimulation of Ca^{2+}-signaling via the T cell receptor/CD3 complex, indicating that a functional $NAADP^+$ Ca^{2+}-release system is essential for T-lymphocyte Ca^{2+}-signaling.

Key words: cyclic ADP-ribose • inositol 1,4,5-trisphosphate • T cell activation • signal transduction • ryanodine receptor

Introduction

Activation of T-lymphocytes via the T cell receptor/CD3 (TCR/CD3)[1] complex results in multiple intracellular signaling pathways (Kennedy et al., 1999). Among these pathways, an elevation of $[Ca^{2+}]_i$ (intracellular Ca^{2+}-concentration) is essential for proliferation and clonal expansion (reviewed in Guse, 1998). The increase of $[Ca^{2+}]_i$ in T cells consists of calcium release from intracellular stores, and, as a major source for the long-lasting Ca^{2+}-signal observed in T cells, subsequent entry of calcium through specific calcium channels in the plasma membrane (reviewed in Guse, 1998). Ca^{2+}-release is activated by the calcium mobilizing second messengers D-*myo*-inositol 1,4,5-trisphosphate($Ins(1,4,5)P_3$) and cyclic ADP-ribose (cADPR). Recent work indicates that $Ins(1,4,5)P_3$ primarily acts during the initial phase of Ca^{2+}-signaling in T cells, whereas cADPR is essentially involved in the sustained phase of Ca^{2+}-signaling (Guse et al., 1999).

Besides $Ins(1,4,5)P_3$ and cADPR, another Ca^{2+}-mobilizing natural compound, nicotinic acid adenine dinucleotide phosphate ($NAADP^+$) was introduced (Chini et al., 1995; Lee and Aarhus, 1995). $NAADP^+$ was originally discovered as a contaminant of commercial nicotinamide adenine dinucleotide phosphate ($NADP^+$) preparations; such preparations could also be enriched in $NAADP^+$ content by alkaline treatment (Clapper et al., 1987). Very low concentrations of $NAADP^+$ in the range of 10–50 nM were shown to effectively release Ca^{2+} from intracellular stores of selected invertebrate and mammalian cell types, such as sea urchin eggs (Lee and Aarhus, 1995), ascidian oocytes (Albrieux et al., 1998), and mouse pancreatic acinar cells (Cancela et al., 1999). $NAADP^+$-mediated Ca^{2+}-release was not sensitive to the cADPR antagonist, $8\text{-}NH_2$-cADPR; the $Ins(1,4,5)P_3$ antagonist, heparin (Lee and Aarhus, 1995); or to the antagonists of ryanodine receptors (RyR), procaine or ruthenium red (Chini et al., 1995). Together, with the lack of cross-desensitization observed between the $NAADP^+/Ca^{2+}$-release system on one hand, and the

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[1]*Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; cADPR, cyclic ADP-ribose; $Ins(1,4,5)P_3$, D-*myo*-inositol 1,4,5-trisphosphate; $InsP_3$-R, $Ins(1,4,5)P_3$ receptor; $Ins(1,4,6)PS_3$, D-*myo*-inositol 1,4,6-trisphosphorothioate; $NAADP^+$, nicotinic acid adenine dinucleotide phosphate; $NADP^+$, nicotinamide adenine dinucleotide phosphate; RyR, ryanodine receptor(s); TCR/CD3, T cell receptor/CD3.

cADPR/- or the Ins(1,4,5)P_3/Ca^{2+}-release systems on the other hand (Chini et al., 1995; Lee and Aarhus, 1995), these data indicate that the $NAADP^+$-dependent Ca^{2+}-release system is different from the two others. Although the receptor for $NAADP^+$ has not yet been identified, unspecific effects of $NAADP^+$ are largely unlikely since concrete structural requirements for $NAADP^+$-mediated Ca^{2+}-release were demonstrated in sea urchin eggs, e.g., the NH_2-group at position 6 of the adenine ring or the phosphate group at the 2′-position of the ribose are necessary (Lee and Aarhus, 1997). The latter can be replaced by a 3′-phosphate or a 2′,3′-cyclic phosphate, but this alteration resulted in a weaker Ca^{2+}-release activity (Lee and Aarhus, 1997). Further characteristic properties of $NAADP^+$-mediated Ca^{2+}-release in sea urchin eggs are a unique self inactivation or self desensitization process (Aarhus et al., 1996; Genazzani et al., 1996), and Ca^{2+}-release from a thapsigargin-insensitive Ca^{2+}-pool (Genazzani and Galione, 1996).

The fact that recent reports indicated a role for $NAADP^+$ in Ca^{2+}-signaling of mammalian cells (Bak et al., 1999; Cancela et al., 1999) prompted us to study its effects in human Jurkat T cells. We report here that $NAADP^+$ specifically and dose-dependently stimulated Ca^{2+}-signaling when microinjected into intact Jurkat T cells. Furthermore, we show that self inactivation of the $NAADP^+$/Ca^{2+}-release system almost completely inhibited Ins(1,4,5)P_3- or cADPR-mediated Ca^{2+}-signaling. Most importantly, we demonstrate that self inactivation of the $NAADP^+$/Ca^{2+}-release also completely antagonized Ca^{2+}-signaling mediated by ligation of the TCR/CD3-complex.

Materials and Methods

Reagents

cADPR, 8-OCH_3-cADPR, and D-*myo*-inositol 1,4,6-trisphosphorothioate (Ins(1,4,6)PS_3) were synthesized exactly as described (Ashamu et al., 1995; Murphy et al., 2000), purified by anion-exchange chromatography on Q-Sepharose, and used as their triethylammonium salts. Purity of ligands was assessed by ^1H and ^{31}P NMR spectroscopy, mass spectroscopy, and, when appropriate, HPLC. $NAADP^+$ and $NADP^+$ were purchased from Sigma-Aldrich. The purity of $NAADP^+$ was described by the manufacturer to be ~95%; this was confirmed by reverse phase HPLC using the method of da Silva et al. (1998). Fura 2/AM was obtained from Calbiochem. Anti-CD3 mAb OKT3 was purified from hybridoma supernatant on protein G–Sepharose.

Cell Culture

Jurkat T lymphocytes (subclone JMP) were cultured in RPMI 1640 medium containing the following additions: glutamax I, Hepes (20 mM, pH 7.4), NCS (7.5%), penicillin (100 U/ml), and streptomycin (50 μg/ml; all obtained from Life Technologies). The cells were cultured at 37°C in a humidified atmosphere in the presence of 5% CO_2.

Ratiometric Ca^{2+} Imaging

Batches of 10^7 Jurkat T cells were loaded with Fura2/AM as described (Guse et al., 1993). Fura2-loaded cells (10^7 cells/5 ml) were kept at room temperature until use. Glass coverslips were coated first with BSA (5 mg/ml), and then with poly-L-lysine (0.1 mg/ml). Small chambers consisting of a rubber O-ring were sealed on the coverslips by silicon grease. Then, 90 μl of extracellular buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM Hepes, 1 mM NaH_2PO_4, 5.5 mM glucose, pH 7.4) was added, followed by addition of 10 μl cell suspension. The coverslip was mounted on the stage of an inverted microscope (Axiovert 100, ZEISS). Ratiometric Ca^{2+} imaging was performed using a PhotoMed/Photon Technology (Wedel) digital imaging system built around the Axiovert 100 microscope. Illumination at 340 and 380 nm was carried out using a chopper/optical filter system. Images were captured by an intensified CCD camera (type C2400-77; spatial resolution: 525 × 487 pixel; Hamamatsu) and stored as individual 340 and 380 images on hard disk. Sampling rate was usually 5 s for a pair of images (340 and 380 nm) using 100-fold magnification. Data analysis was performed off-line using PhotoMed/Photon Technology (Wedel) Image master analysis software. Ratio images (340/380) were constructed pixel by pixel, and changes in the ratio over time were measured by applying regions-of-interest on individual cells. Finally, ratio values were converted to Ca^{2+}-concentrations by external calibration.

Microinjection Experiments

Parallel Ca^{2+} imaging and microinjection experiments require a firm attachment of the Jurkat T cells without preactivation of intracellular Ca^{2+}-signaling. This was achieved by the above mentioned coating procedure of the glass coverslips, as detailed earlier (Guse et al., 1997). The cells were kept in a small chamber (100 μl vol) in extracellular buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM Hepes, 1 mM NaH_2PO_4, 5.5 mM glucose, pH 7.4). Compounds to be microinjected were cleared from particles by either filtration through 0.45-μm filters, by centrifugation in an Eppendorf centrifuge at maximal speed for 10 min, or by both. Femtotips II (Eppendorf) were filled with 5 μl of reagent solution and inserted into the semiautomatic microinjection system (Transjector 5246, Micromanipulator 5171, Eppendorf). Injection parameters were: injection pressure, 80 hPa; compensatory pressure, 40 hPa; duration of injection. 0.5 s; velocity of pipette, 700 μm/s; pipette angle, 45°. Injections were performed into the upper part of the cell.

Results

Microinjection of $NAADP^+$ at a pipette concentration as low as 10 nM stimulated repetitive, long-lasting Ca^{2+}-spiking of low amplitude in intact Jurkat T cells, whereas injection of intracellular buffer alone had no effect (Fig. 1, A, B, E, and F). Microinjection of 0.1 or 1 nM $NAADP^+$ was without effect in most of the cells (Fig. 1, C and D, and data not shown). At a pipette concentration of 50 nM $NAADP^+$, an initial, rapidly occurring Ca^{2+}-peak with a high amplitude was observed which turned into gradually lowering oscillations during the first 350–400 s. After this time period, the calcium response changed into a low, but sustained, plateau phase with very small oscillations (Fig. 1, G and H). At pipette concentrations of 100 nM and 1 μM, similar responses were observed (Fig. 1, I–L). However, the peak amplitude of the initial Ca^{2+}-spike declined with increasing $NAADP^+$ concentrations (Fig. 1, J and L), and the decay of the Ca^{2+}-signal was accelerated (Fig. 1 H, J, and L). At 10 μM $NAADP^+$, the Ca^{2+}-response appeared similar to the one at 1 nM (Fig. 1, M and N), whereas at 100 μM $NAADP^+$, no signal was detectable (Fig. 1, O and P). The dose response relationship shows a bell-shaped curve for the initial Ca^{2+}-peak with an optimal $NAADP^+$ concentration at 100 nM (Fig. 2 A). However, only minor changes of the long-lasting Ca^{2+}-signal as measured at 400 s were observed in response to 100 nM $NAADP^+$ (Fig. 2 B). These data indicate that, similar to the few other cellular systems investigated so far (Chini et al., 1995; Lee and Aarhus, 1995; Albrieux et al., 1998; Cancela et al., 1999), $NAADP^+$ at low nanomolar concentrations activates Ca^{2+}-signaling in T cells, whereas micromolar concentrations of $NAADP^+$ rapidly cause self inactivation of the Ca^{2+}-release system.

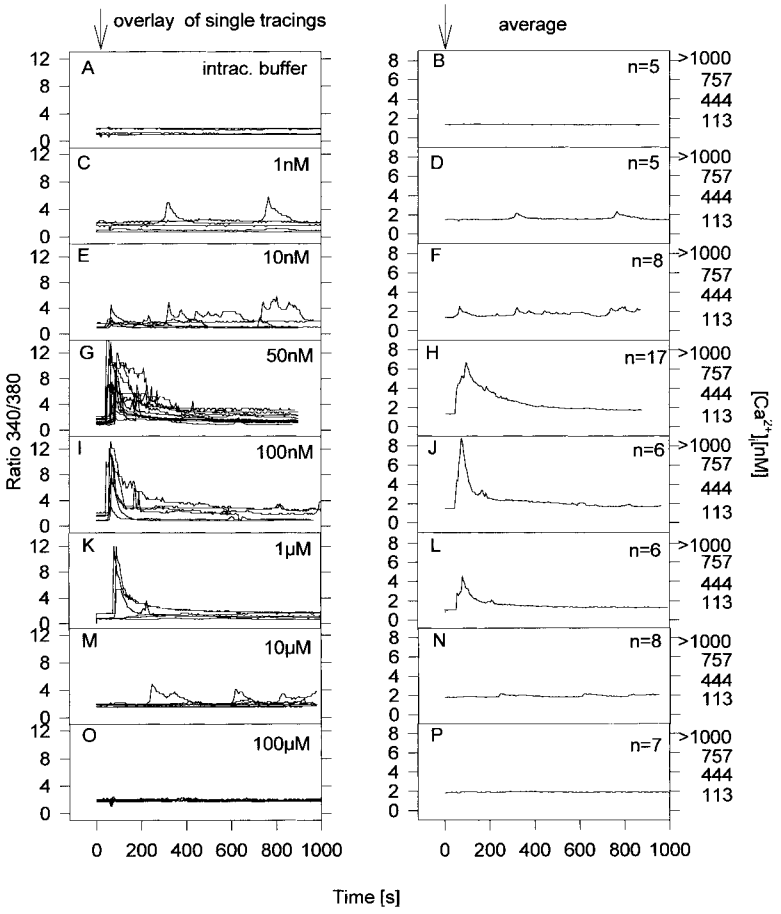

Figure 1. Concentration-response curves of Ca^{2+} signals in single intact T-lymphocytes microinjected with $NAADP^+$. Jurkat T-lymphocytes were loaded with Fura2/AM and Ca^{2+} was measured as detailed in Materials and Methods. Cells were injected as described in Materials and Methods in the presence of 1 mM extracellular Ca^{2+}. Data are presented as overlays of single tracings of individual cells (left). The right shows the corresponding averages from these measurements (number of cells displayed, n = 5–17). As a control, vehicle buffer without $NAADP^+$ was injected (A and B). The time points of microinjection are indicated by arrows.

The high initial Ca^{2+}-spike observed after microinjection of 50 nM $NAADP^+$ was massively reduced when the extracellular Ca^{2+}-concentration was decreased to a nominal Ca^{2+}-free buffer, indicating that Ca^{2+}-entry is involved in the $NAADP^+$-mediated Ca^{2+}-response (data not shown).

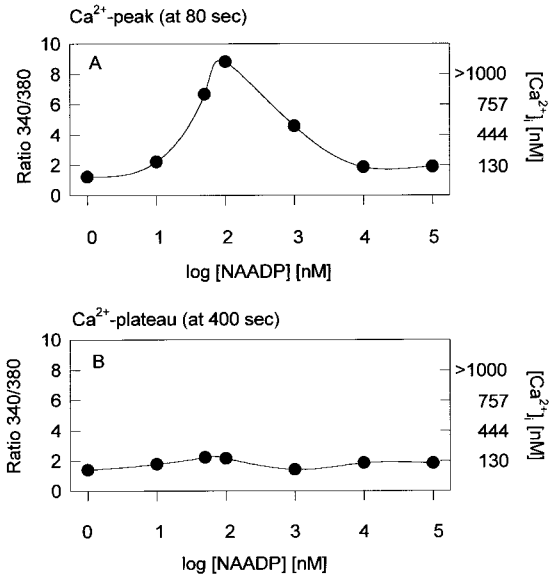

Figure 2. Dose-response curve for $NAADP^+$ in Jurkat T cells. Data from Fig. 1 are shown as mean values (n = 5–17) from time point 80 s (Ca^{2+}-peak; A) or 400 s (Ca^{2+}-plateau; B).

To prove the specificity of the effect of $NAADP^+$ on intracellular Ca^{2+}-signaling in T cells, $NADP^+$ was used in parallel microinjection experiments. $NADP^+$ is a structurally similar molecule, bearing a nicotinamide group instead of the nicotinic acid group. In contrast to $NAADP^+$, microinjection of $NADP^+$ (50 nM) was completely without effect on Ca^{2+}-signaling (Fig. 3, A and B).

The Ca^{2+}-release system that is targeted by $NAADP^+$ has not yet been identified, but work in other cell systems indicates that neither the Ins(1,4,5)P_3 receptor (InsP3-R) nor the RyR are involved (Chini et al., 1995; Lee and Aarhus, 1995). However, both of these classical intracellular Ca^{2+}-release systems have been demonstrated to be essential parts of the Ca^{2+}-signaling machinery of T cells (Jayaraman et al., 1995; Guse et al., 1999). Thus, the next series of experiments was designed to investigate potential interrelations between the $NAADP^+$ system on one hand and both the Ins(1,4,5)P_3 and cADPR systems on the other.

The specific cADPR antagonist 8-OCH_3-cADPR (Guse et al., 1999), when coinjected with an optimal $NAADP^+$ concentration, did not significantly affect $NAADP^+$-mediated Ca^{2+}-signaling (Fig. 4, A and F vs. B and G). However, when a self-desensitizing concentration of $NAADP^+$ (10 µM) was coinjected with a stimulating concentration of cADPR (10 µM), a massive decrease of the cADPR-mediated Ca^{2+}-signal was observed (Fig. 4, C and H vs. E and J). On the other hand, an optimal stimulating concentration of $NAADP^+$ (50 nM) microinjected together with

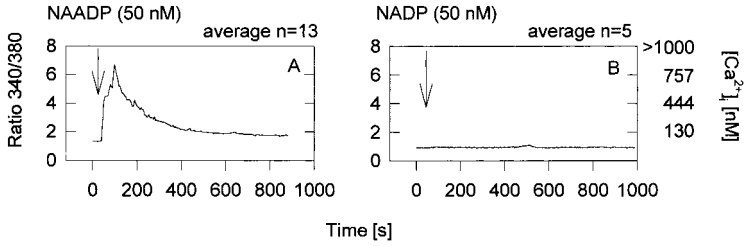

Figure 3. $NADP^+$ does not mediate Ca^{2+}-signaling. Jurkat T-lymphocytes were loaded with Fura2/AM and ratiometric Ca^{2+} imaging, and parallel microinjection in the presence of 1 mM extracellular Ca^{2+} was carried out as detailed under Materials and Methods. Cells were injected with 50 nM $NAADP^+$ (A) or $NADP^+$ (B). Shown are the averages from 13 (A) and 5 (B) cells. The time points of microinjection are indicated by arrows.

cADPR (10 μM) did not significantly change the Ca^{2+}-signals (Fig. 4, D and I vs. E and J). These data indicate that a functional, nondesensitized $NAADP^+/Ca^{2+}$-release system is necessary for cADPR-mediated Ca^{2+}-release.

The specific Ins(1,4,5)P_3 antagonist, Ins(1,4,6)PS_3 (Guse et al., 1997; Murphy et al., 2000), was also coinjected with an optimal $NAADP^+$ concentration. Surprisingly, there was a partial reduction of the initial Ca^{2+}-peak, but also a faster decay of this peak as compared with injection of $NAADP^+$ alone (Fig. 5, A and F vs. B and G). Similar to the cADPR system, there was an almost complete inhibition of Ins(1,4,5)P_3-mediated Ca^{2+}-release when a desensitizing concentration of $NAADP^+$ was coinjected (Fig. 5, C and H). Coinjection of an optimal stimulating concentration of $NAADP^+$, together with Ins(1,4,5)P_3, resulted in a high initial Ca^{2+}-peak (Fig. 5, D and I) that was comparable to the peak observed in response to injection of $NAADP^+$ alone (Fig. 5, B and C), whereas much less oscillatory activity of the cells after the initial peak was observed (Fig. 5, D and I) as compared with Ins(1,4,5)P_3 alone (Fig. 5, E and J). These data also indicate that the Ins(1,4,5)P_3/Ca^{2+}-release system requires a functional nondesensitized $NAADP^+/Ca^{2+}$-release system. Moreover, a part of the Ca^{2+}-signal observed in response to microinjection of $NAADP^+$ alone appears to be mediated by Ins(1,4,5)P_3 (Fig. 5, A, F, B, and G). This may be explained by the coagonistic effect of Ca^{2+} released by $NAADP^+$, which then acts together with basal Ins(1,4,5)P_3 at the $InsP_3$-R; this coagonistic effect of Ca^{2+} at the $InsP_3$-R has been demonstrated previously (Bezprozvanny et al., 1991).

Both the Ins(1,4,5)P_3/Ca^{2+}- and the $cADPR/Ca^{2+}$-

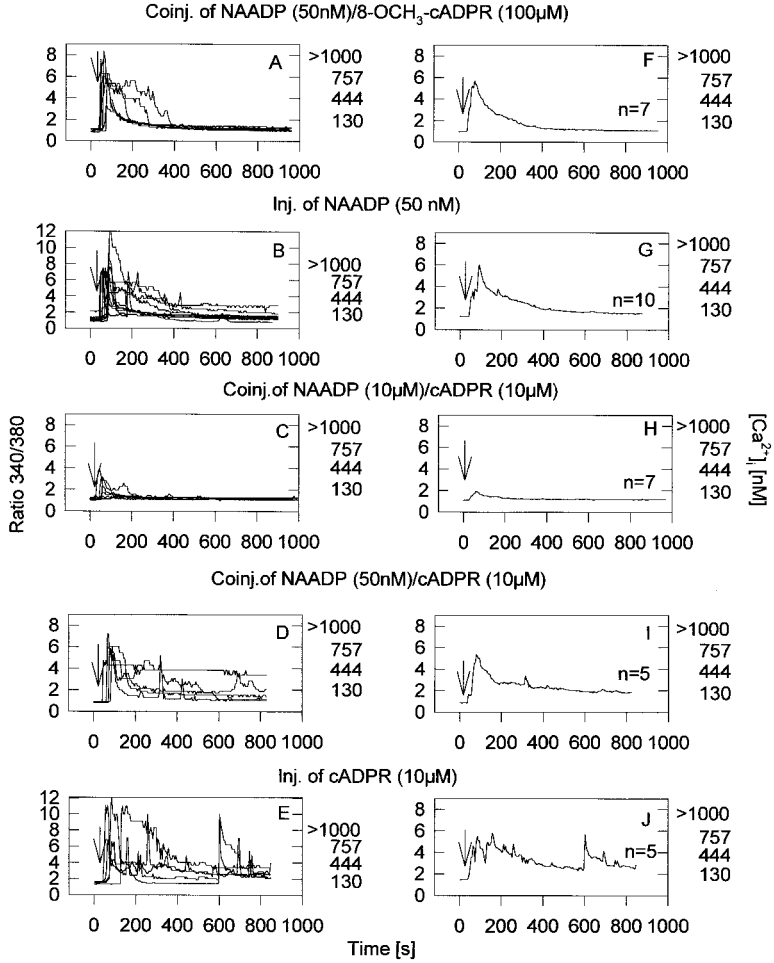

Figure 4. Influence of cADPR and its antagonist 8-OCH_3-cADPR on $NAADP^+$-mediated Ca^{2+}-signaling. Jurkat T-lymphocytes were loaded with Fura2/AM, and ratiometric Ca^{2+} imaging and parallel microinjection in the presence of 1 mM extracellular Ca^{2+} was carried out as detailed under Materials and Methods. Left, Overlays of single tracings of individual cells after injection (A–E); right, demonstrates the corresponding averages from these overlays (F–J). Shown are (n = number of experiments): A/F, coinjection of NAADP (50 nM) and 8-OCH_3-cADPR (100 μM; n = 7); B/G, injection of $NAADP^+$ (50 nM, n = 10); C/H, coinjection of $NAADP^+$ (10 μM) and cADPR (10μM; n = 7); D/I, coinjection of $NAADP^+$ (50 nM) and cADPR (10 μM; n = 5); and E/J, injection of cADPR (10 μM, n = 5).

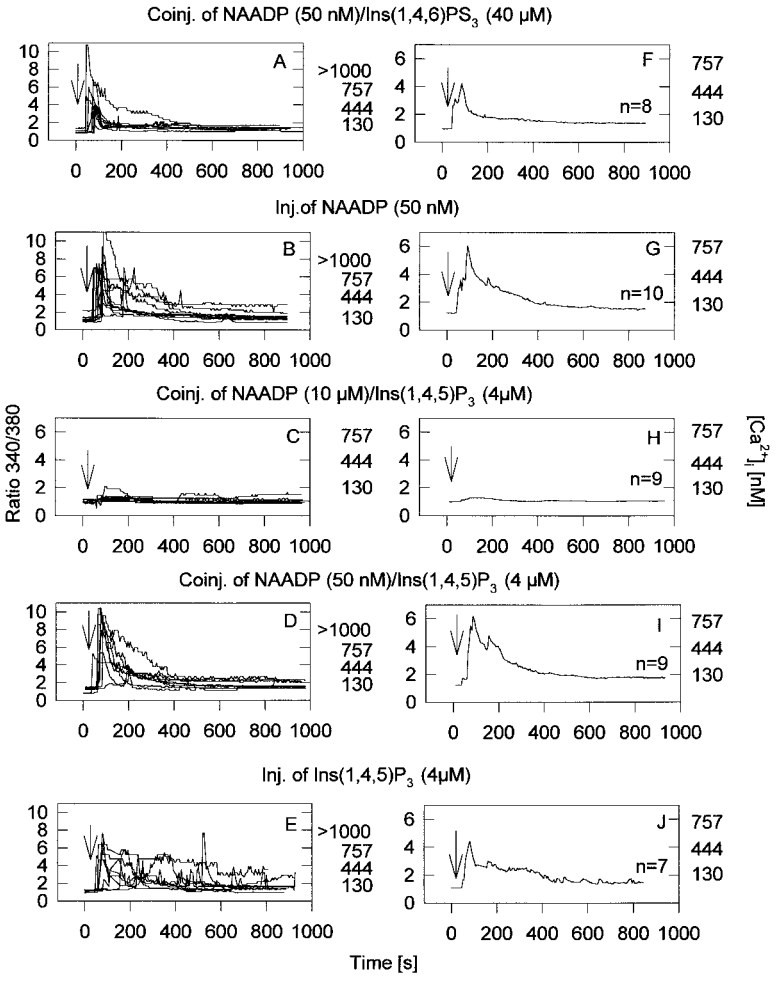

Figure 5. Influence of Ins(1,4,5)P_3 and its antagonist Ins(1,4,6)PS_3 on NAADP$^+$-mediated Ca^{2+}-signaling. Jurkat T-lymphocytes were loaded with Fura2/AM, and ratiometric Ca^{2+} imaging and parallel microinjection in the presence of 1 mM extracellular Ca^{2+} was carried out as detailed under Materials and Methods. Left, Overlays of single tracings of individual cells after injection (A–E); right, demonstrates the corresponding averages from these overlays (F–J). Shown are: A/F, coinjection of NAADP$^+$ (50 nM) and Ins(1,4,6)PS_3 (40 μM; n = 7); B/G, injection of NAADP$^+$ (50 nM; n = 10); C/H, coinjection of NAADP$^+$ (10 μM) and Ins(1,4,5)P_3 (4 μM; n = 9); D/I, coinjection of NAADP$^+$ (50 nM) and Ins(1,4,5)P_3 (4 μM; n = 3); and E/J injection of Ins(1,4,5)P_3 (4 μM; n = 8).

release systems have been published to be essential parts of the Ca^{2+}-signaling machinery of T cells upon stimulation of the TCR/CD3 complex (Jayaraman et al., 1995; Guse et al., 1999). Since the data described above indicate that a functional NAADP$^+$/Ca^{2+}-release system is essential for both Ins(1,4,5)P_3- and cADPR-mediated Ca^{2+}-release, we investigated the effect of NAADP$^+$ on Ca^{2+}-signaling mediated by anti-CD3 mAb OKT3 (Fig. 6). Microinjection of 50 nM NAADP$^+$ before stimulation of the cells by extracellular addition of OKT3 did not significantly change the OKT3-mediated Ca^{2+}-signal (Fig. 6, A and B). However, there was a dramatic inhibition of OKT3-mediated Ca^{2+}-signaling when a desensitizing concentration of NAADP$^+$ was microinjected before stimulation by OKT3 (Fig. 6 C).

Discussion

The main findings of this report are: a dose-dependent and specific effect of NAADP$^+$ in T cell Ca^{2+}-signaling; the strict dependence of both Ins(1,4,5)P_3- and cADPR-mediated Ca^{2+}-release upon a functional NAADP$^+$/Ca^{2+}-release system; and inhibition of Ca^{2+}-signaling mediated by ligation of the TCR/CD3 complex by prior self inactivation of the NAADP$^+$/Ca^{2+}-release system.

In sea urchin eggs, Ca^{2+}-release by NAADP$^+$ was half-maximal between 16 and 30 nM, and showed saturation between ~100 and 400 nM (Chini et al., 1995; Lee and Aarhus, 1995). In ascidian oocytes and mouse pancreatic acinar cells, effective concentrations between 10 and 50 nM were observed (Albrieux et al., 1998; Cancela et al., 1999), although in brain microsomes 1 μM NAADP$^+$ was necessary (Bak et al., 1999). These data fit very well to our current data in Jurkat T cells, the first human cell system where an effect of NAADP$^+$ is reported. Ca^{2+}-mobilizing concentrations were in the range between 10 and 100 nM (Figs. 1 and 2), whereas at concentrations ≥1 μM, partial or complete self inactivation was observed (Figs. 1 and 2).

The self inactivation properties of the NAADP$^+$/Ca^{2+}-release system, at least in sea urchin eggs, appear to be unique as compared with the known Ca^{2+}-mobilizing second messengers, Ins(1,4,5)P_3 and cADPR (Aarhus et al., 1996; Genazzani et al., 1996). Especially the fact that subthreshold concentrations of NAADP$^+$ (2 to 4 nM) almost completely inhibited subsequent Ca^{2+}-release by a high concentration of NAADP$^+$ (Aarhus et al., 1996; Genazzani et al., 1996) indicates that activation of the NAADP$^+$/Ca^{2+}-release system followed by its rapid inactivation can supply the cell with a short pulse of elevated Ca^{2+} only, and that the basal endogenous concentration of NAADP$^+$ must be below a concentration that would permanently inactivate the system, e.g., in sea urchin eggs below 0.1 nM

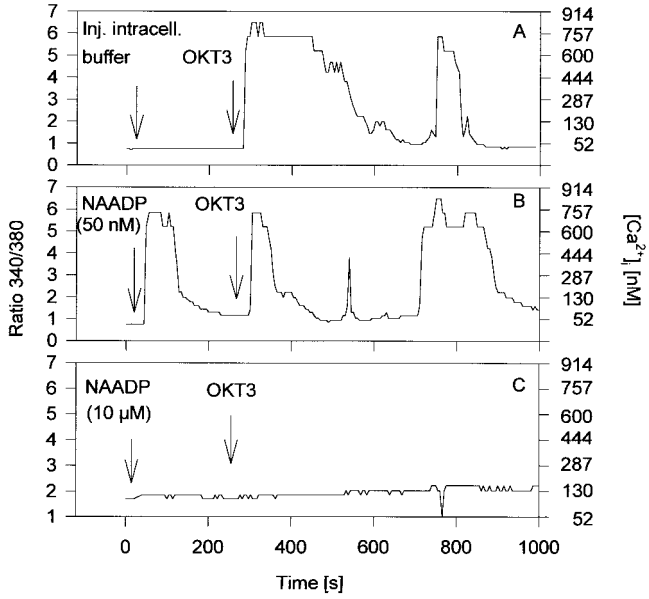

Figure 6. Effect of $NAADP^+$ on OKT3-induced Ca^{2+}-signaling in single Jurkat T-lymphocytes. Jurkat T-lymphocytes were loaded with Fura2/AM, and ratiometric Ca^{2+} imaging and parallel microinjection in the presence of 1 mM extracellular Ca^{2+} was carried out as detailed under Materials and Methods. The cells were injected with different concentrations of $NAADP^+$ and then OKT3 (10 μg/ml) was added. Injection of intracellular buffer (A), $NAADP^+$ (50 nM [B] and 10 μM [C]), and addition of OKT3 is indicated by arrows. Data are presented as a typical tracing from one individual cell; for each condition at least three experiments were carried out.

(Aarhus et al., 1996) or even less (Genazzani et al., 1996). Data in mammalian cell types, pancreatic acinar cells (Cancela et al., 1999), and T cells, however, indicate that low concentrations of $NAADP^+$ do not substantially self-inactivate the system, e.g., microinjections of 10 nM $NAADP^+$ in the majority of cases stimulated long-lasting trains of low-amplitude Ca^{2+}-spikes in T cells (Fig. 1 E), and infusion of 50 nM $NAADP^+$ into acinar cells evoked sustained Ca^{2+}-spiking (Cancela et al., 1999).

To completely unravel the role of $NAADP^+$, mainly to verify (or to falsify) its status as a second messenger, measurement of the endogenous concentration of $NAADP^+$ would be helpful. However, regarding the theoretically expected concentrations of ≤0.1 nM in unstimulated cells and 50–100 nM $NAADP^+$ in stimulated cells, it might be very difficult to develop an analytical system to measure these low concentrations. Our recently developed HPLC systems for the mass determination of $Ins(1,4,5)P_3$ (Guse et al., 1995) and cADPR (da Silva et al., 1998) require $0.5–1 \times 10^8$ cells per sample to measure these compounds in the low micromolar range. To measure basal $NAADP^+$ concentrations a 1,000-fold more sensitive analytical method would be required. Potential methods to achieve this may include labeling of $NAADP^+$ by a fluorescent dye (pre- or postcolumn derivatization) combined with a very sensitive fluorescence detector, e.g., laser-induced fluorescence detection (Rahavendran and Karnes, 1993). Alternatively, a competitive protein binding assay based on a high affinity binding protein for $NAADP^+$ may also be sufficient.

As discussed above, regarding the $NAADP^+/Ca^{2+}$-release system, there are similarities between mouse pancreatic acinar cells and human T cells, e.g., a very similar dose-response relationship for $NAADP^+$, and the fact that self inactivation of the $NAADP^+/Ca^{2+}$-release renders both cell types insensitive to physiological stimulation. However, there are also at least three clear differences between the two cell systems: inhibition of either the $cADPR/Ca^{2+}$-release system or the $Ins(1,4,5)P_3/Ca^{2+}$-release system in pancreatic acinar cells completely blocked $NAADP^+$-mediated Ca^{2+}-signaling (Cancela et al., 1999), whereas similar inhibition protocols were without or almost without effect in T cells; self inactivation of the $NAADP^+/Ca^{2+}$-release system did not influence Ca^{2+}-signaling mediated by infusion of cADPR or $Ins(1,4,5)P_3$ in acinar cells (Cancela et al., 1999), whereas in T cells such self-inactivation of the $NAADP^+/Ca^{2+}$-release system almost completely inhibited subsequent signaling by cADPR or $Ins(1,4,5)P_3$; and in acinar cells, the sustained phase of Ca^{2+}-spiking induced by infusion of cADPR could be blocked by the $Ins(1,4,5)P_3$ antagonist heparin (Thorn et al., 1994), whereas in T cells there was no effect of the $Ins(1,4,5)P_3$ antagonist, $Ins(1,4,6)PS_3$, on cADPR-mediated Ca^{2+}-signaling (Guse et al., 1997).

Using the data obtained from pancreatic acinar cells, Petersen and Cancela (1999) developed a model with the following sequence of events: stimulation of acinar cells by the brain–gut peptide, cholecystokinin, in first instance elevates $NAADP^+$ to nanomolar concentrations. Ca^{2+} released by $NAADP^+$ then serves as a trigger for the Ca^{2+}-induced Ca^{2+}-release mechanism at the RyR. This mechanism, in addition to the stimulatory effect of cADPR on RyR, then amplifies the Ca^{2+}-signal. The increased $[Ca^{2+}]_i$ in concert with $Ins(1,4,5)P_3$ then releases more Ca^{2+} via the $InsP_3$-R (Petersen and Cancela, 1999). Only this last element is measurable as a Ca^{2+}-spike, whereas the trigger and amplifier element, provided by $NAADP^+$ and the Ca^{2+}-induced Ca^{2+}-release mechanism modulated by cADPR, appear to be too small to be detected by patch clamp measurements of the Ca^{2+}-dependent currents (Petersen and Cancela, 1999).

In contrast to acinar cells, in Jurkat T cells $NAADP^+$ produced a substantial Ca^{2+}-spike, even if cADPR- and $Ins(1,4,5)P_3$-antagonists were present (Figs. 4, A and F, and 5, A and F). The second main difference to acinar cells was that in Jurkat T cells, Ca^{2+}-signaling by cADPR and $Ins(1,4,5)P_3$ depended on a functional $NAADP^+/Ca^{2+}$-release system (Figs. 4, C and H, and 5, C and H). Although two different methods were used to detect the Ca^{2+}-spikes: patch clamp measurements of the Ca^{2+}-dependent currents vs. single cell Ca^{2+} imaging using Fura2-loaded cells, this is unlikely to be the reason for the differences observed. Thus, the model developed for the acinar cells (Petersen and Cancela, 1999) needs some modification to fit to the data obtained in Jurkat T cells. In accordance with the acinar cell model, $NAADP^+$ appears to act first in sequence providing trigger-calcium needed for the two other Ca^{2+}-release systems. Because of the experimental difficulties to measure nanomolar concentrations of $NAADP^+$ in cells as discussed above, it is unclear whether

$NAADP^+$ concentrations in fact do increase upon stimulation, or whether $NAADP^+$ stays unaltered in the low nanomolar range keeping the T cell in an excitable state. Experimental evidence for the latter may be obtained by high temporal and spatial resolution Ca^{2+} imaging experiments in Jurkat T cells; preliminary data indicate a basal Ca^{2+}-signaling activity of very low amplitude in nonstimulated cells (Guse, A.H., and S. Heidbrink, unpublished results). However, trigger-calcium provided by $NAADP^+$ further acts in concert with $Ins(1,4,5)P_3$, which is rapidly, but transiently, formed in the first minutes of T cell activation (Brattsand et al., 1990; Ng et al., 1990), and then with cADPR, which is elevated during the sustained phase of T cell Ca^{2+}-signaling (Guse et al., 1999).

However, despite these differences between acinar and T cells the dependence of cholecystokinin receptor–mediated Ca^{2+}-signaling on a Ca^{2+}-trigger supplied by an initial $NAADP^+$-mediated Ca^{2+}-release event to integrate the Ca^{2+}-amplifier, cADPR, and the Ca^{2+}-oscillator, $Ins(1,4,5)P_3$ (Petersen and Cancela, 1999), exactly mirrors the situation observed in Jurkat T cells. As shown in Fig. 6, if the $NAADP^+/Ca^{2+}$-release system was inactivated by high $NAADP^+$ concentrations subsequent quasiphysiological stimulation of the T cell by anti-CD3 mAb did not result in any Ca^{2+}-signaling. This result is in complete accordance with the inhibition of cADPR- or $Ins(1,4,5)P_3$-mediated Ca^{2+}-signaling by coinjection of $NAADP^+$ (Figs. 4, C and H, and 5, C and H) since Ca^{2+}-signaling in T cells critically depends on these two second messengers (Jayaraman et al., 1995; Guse et al., 1999).

More generalized, if the $NAADP^+/Ca^{2+}$-release system acts as the Ca^{2+}-providing trigger, the complex behavior of activation and inactivation opens a multitude of regulatory possibilities: simply by changing their endogenous $NAADP^+$ concentration cells might regulate the status of the $NAADP^+/Ca^{2+}$-release system; e.g., by increasing $NAADP^+$ the $NAADP^+/Ca^{2+}$-release system will become inactivated, and Ca^{2+}-signaling will in turn be completely unresponsive. For T-lymphocytes, such behavior of unresponsiveness to antigenic or mitogenic stimulation is well known as anergy (Jenkins et al., 1987); however, it is less clear which intracellular mechanism is responsibly involved. Our data indicate that the $NAADP^+/Ca^{2+}$-release system with its complex inactivation/activation properties might be such a mechanism underlying anergy in T cells.

From an evolutionary point of view, it is of particular interest that both a very similar dose-response relationship and the self inactivation property of the $NAADP^+/Ca^{2+}$-release have been conserved between sea urchin eggs, ascidian oocytes, and higher eukaryotic cells from pancreas and lymphocytes. This indicates that these two characteristic features are of outstanding importance for the regulation of intracellular Ca^{2+}-signaling in general. One of the important future aspects will be the identification of the molecular target for $NAADP^+$. In addition to the model for pancreatic acinar cells (Cancela et al., 1999; Petersen and Cancela, 1999) in which a separate $NAADP^+$ receptor has been suggested, it might also be possible that $NAADP^+$ acts as a comessenger at the known intracellular Ca^{2+}-release channels, the $Ins(1,4,5)P_3$ receptor and/or the RyR. Along these lines, the fluorescent $1,N^6$-etheno-$NAADP^+$ has been shown to release Ca^{2+} in sea urchin eggs (Lee and Arhus, 1998), and thus, may serve as a tool to identify the receptor for $NAADP^+$.

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