Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent Ca$^{2+}$-mobilizing nucleotide involved in T cell Ca$^{2+}$ signaling (Berg, I., Potter, B. V. L., Mayr, G. W., and Guse, A. H. (2000) J. Cell Biol. 150, 581–588). The objective of this study was to analyze whether the first subcellular Ca$^{2+}$ signals obtained upon NAADP stimulation of T-lymphocytes depend on the functional expression of ryanodine receptors. Using combined microinjection and high resolution confocal calcium imaging, we demonstrate here that subcellular Ca$^{2+}$ signals, characterized by amplitudes between ~30 and 100 nM and diameters of ~0.5 μm, preceded global Ca$^{2+}$ signals. Co-injection of the ryanodine receptor antagonists ruthenium red and ryanodine together with NAADP abolished the effects of NAADP, whereas the d-myo-inositol 1,4,5-trisphosphate antagonist heparin and the Ca$^{2+}$ entry blocker SKF&96365 were without effect. This pharmacological approach was confirmed by a molecular knock-down approach. Jurkat T cell clones with largely reduced expression of ryanodine receptors did not respond to microinjections of NAADP. Taken together, our data suggest that the Ca$^{2+}$ release channel sensitive to NAADP in T-lymphocytes is the ryanodine receptor.

Nicotinic acid adenine dinucleotide phosphate (NAADP) is an endogenous nucleotide in eukaryotic cells and to date represents the most powerful Ca$^{2+}$-releasing compound. This compound was discovered by Lee and co-workers as an impurity of NADP in 1987 (1), and its structure was obtained in 1995 (2).

In contrast to the other known Ca$^{2+}$-releasing compounds, d-myo-inositol 1,4,5-trisphosphate (InsP$_3$) (for review see Ref. 3) and cyclic ADP-ribose (cADPR) (for review see Ref. 4), the Ca$^{2+}$ channel sensitive to NAADP, is still a matter of debate. Pharmacological Ca$^{2+}$ release data obtained in sea urchin egg homogenates suggest that neither the InsP$_3$ receptor nor the ryanodine receptor (RyR) represents the molecular target for NAADP (2). In addition, NAADP appears not to act on the classical rapidly exchanging Ca$^{2+}$ store, the endoplasmic reticulum. Indeed, a NAADP-sensitive Ca$^{2+}$ pool was separated from the cADPR- and InsP$_3$-sensitive one by stratification of sea urchin eggs (5). Subsequently, this store was identified as the reserve granule of eggs, a lysosome-related organelle (6).

Recently, a lysosome-related acidic Ca$^{2+}$ store sensitive to NAADP was also detected in higher eukaryotic cells, e.g. pancreatic acinar cells and the MIN6 pancreatic β-cell line (7), and in rat cortical neurons (8).

Although the pharmacological characterization of NAADP-induced Ca$^{2+}$ signaling in sea urchin eggs resulted in the conclusion that a novel Ca$^{2+}$ channel unrelated to the known intracellular Ca$^{2+}$ release channels is involved, a number of reports from heart and skeletal muscle and pancreatic acinar cells suggest that RyR are the Ca$^{2+}$ channels mediating the effect of NAADP (9–11).

Accordingly, different models resulting from these conflicting data have been postulated. The two-pool model (12) consists of two separate Ca$^{2+}$ pools: a lysosome-related Ca$^{2+}$ pool with the novel NAADP receptor giving rise to spatiotemporally restricted trigger Ca$^{2+}$ and an endoplasmic reticulum-related Ca$^{2+}$ pool with InsP$_3$, receptors and RyR, which then respond to the trigger Ca$^{2+}$ by Ca$^{2+}$-induced Ca$^{2+}$ release (CICR). The other model reduced the number of Ca$^{2+}$ channels sensitive to NAADP to the RyR but postulated different binding proteins for NAADP and cADPR, mediating their effects at the RyR (11). We have recently shown that global Ca$^{2+}$ signaling induced by NAADP in human Jurkat T-lymphocytes depends on functional expression of RyR and also on Ca$^{2+}$ entry (13).

Because that report was compatible with both models, the present study was conducted to analyze the very initial subcellular Ca$^{2+}$ release events observed upon NAADP stimulation and, in particular, to understand whether the RyR is necessary also for these spatiotemporally restricted Ca$^{2+}$ signals.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fetal calf serum (tetracycline-free) was obtained from Biochrom (Berlin, Germany). Fura-2/AM, ionomycin, ryanodine, SKF 96365, and ruthenium red were purchased from Calbiochem. NAADP and heparin were supplied by Sigma. cADPR was obtained from Biolog (Bremen, Germany).

**Cell Culture**—Jurkat T-lymphocytes (clone JMP) were cultured as described previously (14). Tet-On Jurkat T cell clones stably transfected with pTRE2-enhanced green fluorescent protein/E2 (abbreviated as clone E2), pTRE2-511/25 (abbreviated as clone 25), and pTRE2-240/10 (abbreviated as clone 10) were cultured in RPMI 1640 medium supplemented with Glutamax I, 10% (v/v) fetal calf serum (free of tetracycline, Biochrom), 25 mM HEPEs, 1 mM sodium pyruvate, 100 units/ml penicillin, 50 μg/ml streptomycin, 50 μg/ml hygromycin, and 400 μg/ml G418-sulfate (15).
Ratiometric Ca\textsuperscript{2+}/H11001 Imaging—The cells were loaded with Fura-2/AM as described by Kunerth et al. (16) and kept in the dark at room temperature until use. Thin glass coverslips (0.1 mm) were coated with bovine serum albumin (5 mg/ml) and poly-L-lysine (0.1 mg/ml). Silicon grease was used to seal small chambers consisting of a rubber O-ring on the glass coverslips. 60 μl of buffer A containing 140 mM NaCl, 5 mM KCl, 1 mM MgSO\textsubscript{4}, 1 mM CaCl\textsubscript{2}, 1 mM NaH\textsubscript{2}PO\textsubscript{4}, 5.5 mM glucose, and 20 mM HEPES, pH 7.4, and a 40-μl cell suspension (2 × 10\textsuperscript{6} cells/ml) suspended in the same buffer were added into the small chamber (16).

The coverslip with cells slightly attached to the bovine serum albumin/poly-L-lysine coating was mounted on the stage of a fluorescence microscope (Leica DM IRE2).

Ratiometric Ca\textsuperscript{2+} imaging was performed as described recently (16). We used an Improvision imaging system (Tubingen, Germany) built around the Leica microscope at 100-fold magnification. Illumination at 340 and 380 nm was carried out using a monochromator system (Polychromator IV, TILL Photonics, Graefeling, Germany). Images were taken with a gray-scale CCD camera (type C4742-95-12ER;
Hamamatsu, Enfield, United Kingdom) operated in 8-bit mode. The spatial resolution was 512 × 640 pixel at 100-fold magnification. Camera exposure times were 12 (at 340 nm) and 4 ms (at 380 nm). The acquisition rate was ~1 ratio m 160 ms. Raw data images were stored on a hard disk. Confocal Ca$^{2+}$ images were obtained by off-line no-neighbor deconvolution using the volume deconvolution module of the Openlab software as described recently for 3T3 fibroblasts (17). The deconvolved images were used to construct ratio images (340/380). Finally, ratio values were converted to Ca$^{2+}$ concentrations by external calibration. To reduce noise, ratio images were subjected to median filtering (3 × 3) as described previously (16). Data processing was performed using Openlab software, version 1.7.8, 3.0.9, or 3.5.2 (Improvisation, Tubingen, Germany).

Microinjection—Microinjections were carried out as described previously (16) using a transjector type 5171, Eppendorf-Netheler-Hinz, Hamburg, Germany) with Femtotips II as pipettes. NAADP and/or ruthenium red were diluted to their final concentration in intracellular buffer (20 mM HEPES, 110 mM KCl, 2 mM MgCl$_2$, 5 mM KH$_2$PO$_4$, 10 mM NaCl, pH 7.2) and filtered (0.2 μm) before use. To avoid any contamination of Ca$^{2+}$ in the solution to be injected, a small amount of Chelex resin beads was added. Injections were made with the semiautomatic mode of the system with the following instrumental settings: injection pressure, 60 (clone JMP) or 40 hectoPascals (clones E2, 10, and 25); compensatory pressure, 30 (clone JMP) or 25 hectoPascals (clones E2, 10, and 25); injection time, 0.5 (clone JMP) or 0.3 s (clone E2, 10, and 25), and velocity of the pipette, 700 μm/s. Under such conditions, the injection volume was 1–1.5% cell volume (19).

RESULTS

Subcellular Ca$^{2+}$ signaling upon microinjection of 100 nM NAADP into Jurkat T-lymphocytes (clone JMP) remained almost unchanged for a very short, initial period of a few hundred milliseconds (Fig. 1A; time point 37.730 s). Characteristic NAADP-induced subcellular signals were observed (Fig. 1A; time point 37.903 s). These initial signals usually were localized close to the site of injection and rapidly converged into a larger signal still remaining mainly in a zone close to the plasma membrane (Fig. 1A; time point 38.077 s). The signal spread in a ring-like structure before it also arrived in the central part of the cell (Fig. 1A; time points 38.252 and 38.433 s). Thus, 100 nM NAADP in the microinjection pipette induced a very rapid global response leaving just a very limited period of time, usually <1.5 s, to analyze subcellular Ca$^{2+}$ signaling events. To expand this pacemaker phase, the pipette concentration of NAADP was reduced to 30 nM, a concentration still sufficient to induce global Ca$^{2+}$ signaling (20). Under these conditions, subcellular Ca$^{2+}$ signals of very small diameter and amplitude were observed (Fig. 1B; time point 40.562 and 41.260 s). As for the 100 nM NAADP injections, the characteristic ring-like signaling structure became visible before global Ca$^{2+}$ signaling started (Fig. 1B; time point 42.303 and 43.531 s). Importantly, when intracellular buffer, the vehicle for NAADP injections, was microinjected into Jurkat T-lymphocytes, no alterations in subcellular Ca$^{2+}$ signaling were observed (Fig. 1C). These control data, together with the fact that in the very initial period after microinjection almost no changes in Ca$^{2+}$ signaling were observed, suggest that (i) the subcellular signals were caused by NAADP and that (ii) the temporal resolution of image acquisition was sufficient to analyze the very initial NAADP-induced Ca$^{2+}$ signals.

![Image](263x39 to 563x262)

**Fig. 3.** Inhibition of subcellular Ca$^{2+}$ signals induced by NAADP by co-injection of ruthenium red. Jurkat T cells (subclone JMP) were loaded with Fura-2/AM and subjected to combined Ca$^{2+}$ imaging and microinjection as detailed under “Experimental Procedures.” A and B, analysis of global [Ca$^{2+}$] ($\Delta$) or local [Ca$^{2+}$] ($\Delta$) in response to injection of NAADP (30 nM), NAADP (30 nM) plus ruthenium red (RUred; 10 μM), or intracellular buffer. Local responses were recorded in the cytosol close to the pm, in the cyt, or in the nuc and expressed as the difference between the maximal peak amplitude and the amplitude of the surrounding area as shown for an example in Fig. 3. Pipette concentrations of inhibitors are as follows: ruthenium red (RUred) (10 μM); ry-anodine (Rya) (20 mM); and heparin (Hep) (1 mg/ml) (note that these concentrations are diluted 1:50–1:100 in the cytosol). The cells were preincubated with SKF 96365 (30 μM) for 15 min in the extracellular medium. Peak amplitudes are given as the mean ± S.E. (n = 5–14 cells for each condition); p values were obtained from the Student’s t test as indicated.

![Image](59x625 to 306x598)

**Fig. 4.** Effect of various Ca$^{2+}$ signaling inhibitors on the amplitude of NAADP evoked subcellular Ca$^{2+}$ signals. Local responses were recorded in the cytosol close to the pm, in the cyt, or in the nuc and expressed as the difference between the maximal peak amplitude and the amplitude of the surrounding area as shown for an example in Fig. 3. Pipette concentrations of inhibitors are as follows: ruthenium red (RUred) (10 μM); ry-anodine (Rya) (20 mM); and heparin (Hep) (1 mg/ml) (note that these concentrations are diluted 1:50–1:100 in the cytosol). The cells were preincubated with SKF 96365 (30 μM) for 15 min in the extracellular medium. Peak amplitudes are given as the mean ± S.E. (n = 5–14 cells for each condition); p values were obtained from the Student’s t test as indicated.
A magnified view of the images showing NAADP-induced subcellular Ca\(^{2+}\)/H\(\text{11001}\) signals revealed that, in case of the 100 nM injection, the signals were more concentrated in one part of the cell, almost merging into one much bigger Ca\(^{2+}\)/H\(\text{11001}\) signal (Fig. 2A, arrow; diameter of the merged signal 2 \(\mu\)m). However, individual small Ca\(^{2+}\) signals with diameters characteristic for Ca\(^{2+}\) quarks (diameter 0.5 \(\mu\)m) were still observed in close vicinity to the bigger signal (Fig. 2A, arrowheads). Upon 30 nM NAADP microinjections, mainly the smaller Ca\(^{2+}\) signals were observed (Fig. 2B, arrowheads).

Very recently, we have shown that global Ca\(^{2+}\) signals evoked by microinjection of NAADP in T cells were completely dependent on functional expression of RyR (13). However, these data left open the possibility that NAADP may initially activate a Ca\(^{2+}\) channel different from the RyR, giving rise to local Ca\(^{2+}\) signals, which then by the process of CICR would trigger RyR, according to the 2-pool model proposed originally by Cancela et al. (12). To distinguish between the 2-pool model and a model where RyR directly (or via an additional binding protein) responds to NAADP (11), subcellular NAADP-induced Ca\(^{2+}\) signals as described in Figs. 1 and 2 were analyzed under further experimental conditions. Ca\(^{2+}\) signaling was analyzed in T cells co-injected with the following: (i) NAADP and the RyR antagonists ruthenium red and ryanodine; (ii) NAADP and the InsP\(_3\) antagonist heparin; or (iii) NAADP and the Ca\(^{2+}\) entry blocker SKF 96365.

As reported previously (13), the global Ca\(^{2+}\) signal observed upon NAADP injection was completely abolished when ruthenium red was co-injected (Fig. 3A). An analysis of small subcellular regions of interest (ROI) set either close to the plasma membrane (pm), the cytosol (cyt), or the nucleus (nuc) revealed an oscillatory behavior of the local [Ca\(^{2+}\)] (Fig. 3B). Importantly, the amplitude of local Ca\(^{2+}\) signals increased with time in the presence of NAADP (Fig. 3B, black tracings). In contrast, when NAADP was co-injected with ruthenium red, the subcellular signals remained at similar amplitudes compared with buffer injections (Fig. 3B, red and blue tracings). Quantitative evaluation of an array of microinjection and preincubation data revealed the following. Co-injection of the antagonists of RyR, ruthenium red and ryanodine (the latter at a high inhibitory concentration), completely blocked subcellular Ca\(^{2+}\) signals evoked by NAADP, regardless of the subcellular localization of the signals (Fig. 4). In contrast, neither co-injection of the InsP\(_3\) antagonist heparin nor preincubation with the Ca\(^{2+}\) entry blocker SKF 96365 antagonized subcellular signals evoked by NAADP (Fig. 4). Rather, a weak stimulatory effect of both heparin microinjection and preincubation with SKF 96365 in the absence and presence of NAADP (co-)injection was ob-

![Comparison of the spatiotemporal characteristics of subcellular Ca\(^{2+}\) signals induced by NAADP or by cADPR.](image-url)

**FIG. 5.** Comparison of the spatiotemporal characteristics of subcellular Ca\(^{2+}\) signals induced by NAADP or by cADPR. Jurkat T cells (subclone JMP) were loaded with Fura-2/AM and subjected to combined Ca\(^{2+}\) imaging and microinjection as detailed under “Experimental Procedures.” A, pseudocolor images displaying spatiotemporal development of Ca\(^{2+}\) signaling in characteristic cells injected with NAADP (30 nM) or cADPR (100 \(\mu\)M). B, local [Ca\(^{2+}\)] in response to injection of NAADP (30 nM) or cADPR (100 \(\mu\)M) recorded in the cytosol close to the pm, in the cyt, or in the nuc and expressed as the difference between the maximal peak amplitude and the amplitude of the surrounding area. Arrows indicate time point of injection (solid for NAADP; dashed for cADPR). Data are representative of 5–9 individual cells analyzed for each condition.
served (Fig. 4). The weak agonistic effect of heparin at the RyR and the weak Ca\(^{2+}\)-mobilizing properties of SKF 96365 are well documented (21, 22) and thus explain the observed effects. Autoinactivation of the NAADP signaling system by injection of a high concentration of NAADP resulted in signal amplitudes comparable to buffer injections. In addition, subcellular Ca\(^{2+}\) signals evoked by the established RyR agonist cADPR were fully blocked by this procedure (Fig. 4).

Analysis of the spatiotemporal pattern of Ca\(^{2+}\) signaling evoked by NAADP compared with the pattern induced by the established RyR agonist cADPR did not result in significant differences (Fig. 5) as can be seen from the images (Fig. 5A) and the ROI tracings close to the pm, in the cyt, or in the nuc (Fig. 5B).

Although the pharmacological data and the spatiotemporal characteristics of NAADP-mediated subcellular Ca\(^{2+}\) signals suggest involvement of RyR, the use of inhibitors may be misleading due to insufficient specificity. Therefore, in a second independent approach, microinjection and imaging experiments were conducted in RyR knock-down T cell clones (13, 15, 18). These RyR knock-down Jurkat T cell clones were obtained by stable transfection with plasmids expressing antisense RNA against RyR (15). Clone 25 expressed antisense RNA specific for the type 3 RyR, whereas clone 10 expressed an antisense RNA fragment targeting all three types of RyR. As control, a Jurkat T cell clone with stable expression of an antisense RNA fragment directed against enhanced green fluorescent protein. Largely reduced expression of RyR on the protein level was analyzed by Western blot experiments and is documented by Langhorst et al. (13) and Schwarzmann et al. (15).

Microinjection of NAADP into T cells of the control clone E2 resulted in comparable subcellular signals as described above for the Jurkat T cell clone JMP (Fig. 6A). In stark contrast, no changes in subcellular Ca\(^{2+}\) signaling were observed in the RyR knock-down T cells, neither in the pan knock-down clone 10 (Fig. 6B) nor in the type 3-specific knock-down clone 25 (Fig. 6C). Quantitative evaluation of individual ROI set close beneath the pm, in the cyt, or in the nuc resulted in an even clearer picture (Fig. 7) compared with the experiments described in Figs. 3 and 4. Rapidly after microinjection of NAADP, the amplitude of subcellular Ca\(^{2+}\) signals in all three ROIs increased in control clone E2 but remained almost unchanged in knock-down clones 10 and 25 (Fig. 7B). Quantitative evaluation of such experiments showed that NAADP induced a statistically significant increase of the signal amplitude in all three ROI in control clone E2, whereas in RyR knock-down clones 10 and 25, no significant differences to buffer injections were observed.

**DISCUSSION**

Here we report that the initial subcellular Ca\(^{2+}\) signals induced by microinjection of NAADP depend on the functional expression of RyR in human T cells. Cancela et al. (23) were the first to show that Ca\(^{2+}\) spiking evoked by NAADP could be efficiently blocked by an inhibitory concentration of ryanodine (23). Obviously influenced by the fact that inhibition of RyR did not affect NAADP-induced Ca\(^{2+}\) release in the sea urchin egg system (2), Cancela et al. (23) interpreted their data not as a direct effect but rather as inhibition of CICR via RyR secondary to the "trigger Ca\(^{2+}\)" provided by a separate and novel receptor/Ca\(^{2+}\) release channel sensitive to NAADP.

In a recent report (13), we demonstrated that both RyR inhibition and the down-regulation of its expression almost completely abolished NAADP-mediated global Ca\(^{2+}\) signaling in T cells. These data, although pronouncing the role of RyR in NAADP-induced Ca\(^{2+}\) signaling in T cells, were still compatible with a separate and novel receptor/Ca\(^{2+}\) release channel sensitive to NAADP. Accordingly, the task of this putative channel would then be to initiate CICR via RyR by evoking local pacemaker Ca\(^{2+}\) signals. The alternative model is simpler and has been proposed for heart and skeletal muscle and for pancreatic acinar cells (9–11). NAADP either directly or via a specific binding protein binds to RyR to induce opening of its Ca\(^{2+}\) channel. In the experiments described in the current report, inhibition by the antagonists of RyR, ryanodine (at high concentration) or ruthenium red, abolished any NAADP-mediated subcellular Ca\(^{2+}\) signals above intrinsic background. In
mediated subcellular Ca\textsuperscript{2+} signals were also obtained in RyR knock-down T cell clones. Thus, the relevance of RyR for these subcellular Ca\textsuperscript{2+} signals evoked by NAADP was confirmed by a completely independent molecular approach.

The NAADP-mediated subcellular Ca\textsuperscript{2+} signals analyzed were very small in diameter (starting from ~0.5 μm) and also small in amplitude (average between 50 and 100 nm), indicating that they may represent an opening of small clusters of RyR, as proposed recently (24). Microinjection of the established RyR agonist cADPR produced subcellular Ca\textsuperscript{2+} signals with similar spatiotemporal characteristics compared with NAADP, further indicating that both adenine nucleotides act on the same target Ca\textsuperscript{2+} channel.

Taken together, our data suggest that RyR are, in addition to their central role in the global Ca\textsuperscript{2+} signals, also responsible for the very initial subcellular Ca\textsuperscript{2+} signals evoked by NAADP in human T-lymphocytes. Our data do not rule out that NAADP-sensitive RyR may be expressed in acidic lysosomal Ca\textsuperscript{2+} stores in T-lymphocytes or that a specific binding protein for NAADP mediating its effect at the RyR exists. However, evidence for a novel NAADP-sensitive Ca\textsuperscript{2+} channel in T cells was not obtained using state-of-the-art Ca\textsuperscript{2+}-imaging technology.

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