NAADP Mobilizes Calcium from the Endoplasmic Reticular Ca\(^{2+}\) Store in T-lymphocytes*

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The target calcium store of nicotinic acid adenine dinucleotide phosphate (NAADP), the most potent endogenous calcium-mobilizing compound known to date, has been proposed to reside in the lysosomal compartment or in the endo/sarcoplasmic reticulum. This study was performed to test the hypothesis of a lysosomal versus an endoplasmic reticular calcium store sensitive to NAADP in T-lymphocytes. Pretreatment of intact Jurkat T cells with glycylyl-phenylalanine 2-naphthylamide largely reduced staining of lysosomes by LysoTracker Red and abolished NAADP-induced Ca\(^{2+}\) signaling. However, the inhibitory effect was not specific since Ca\(^{2+}\) mobilization by d-myo-inositol 1,4,5-trisphosphate and cyclic ADP-ribose was abolished, too. Bafilomycin A1, an inhibitor of the lysosomal H\(^{+}\)-ATPase, did not block or reduce NAADP-induced Ca\(^{2+}\) signaling, although it effectively prevented labeling of lysosomes by LysoTracker Red. Further, previous T cell receptor/CD3 stimulation in the presence of bafilomycin A1, assumed to block refilling of lysosomal Ca\(^{2+}\) stores, did not antagonize subsequent NAADP-induced Ca\(^{2+}\) signaling. In contrast to bafilomycin A1, emptying of the endoplasmic reticulum by thapsigargin almost completely prevented Ca\(^{2+}\) signaling induced by NAADP. In conclusion, in T-lymphocytes, no evidence for involvement of lysosomes in NAADP-mediated Ca\(^{2+}\) signaling was obtained. The sensitivity of NAADP-induced Ca\(^{2+}\) signaling toward thapsigargin, combined with our recent results identifying ryanodine receptors as the target calcium channel of NAADP (Dammermann, W., and Guse, A. H. (2005) J. Biol. Chem. 280, 21394–21399), rather suggest that the target calcium store of NAADP in T cells is the endoplasmic reticulum.

Nicotinic acid adenine dinucleotide phosphate (NAADP)\(^3\) was discovered by Lee et al. (1) as endogenous nucleotide with Ca\(^{2+}\)-mobilizing properties. In comparison with the Ca\(^{2+}\)-mobilizing second messengers d-myo-inositol 1,4,5-trisphosphate (InsP\(_3\)) and cyclic ADP-ribose (cADPR) that require micromolar concentrations for activity in mammalian cells (2, 3), NAADP acts at low nanomolar concentrations and displays a bell-shaped concentration-response curve in many different eukaryotic cell types (4–6). Although InsP\(_3\) and cADPR are firmly established as second messengers, receptor-mediated formation of NAADP has yet been shown in a limited number of cellular systems only (7–9).

The search for the target organelle and the molecular receptor for NAADP has attracted much attention in the last years. Lee et al. (10) demonstrated different subcellular localizations of the NAADP-sensitive Ca\(^{2+}\) pool versus the InsP\(_3\)- and cADPR-sensitive Ca\(^{2+}\) pools in stratified sea urchin eggs. This NAADP-sensitive Ca\(^{2+}\) pool was identified as the reserve granule in sea urchin eggs, an organelle related to lysosomes (11). In addition, pharmacological and partial biochemical characterization suggests expression of a novel NAADP receptor/Ca\(^{2+}\) channel in sea urchin eggs (1, 12–15).

In a few higher eukaryotic cell types, evidence for the involvement of ryanodine receptors (RyR) in NAADP-mediated Ca\(^{2+}\) signaling has been published. NAADP activated purified RyR from heart and skeletal muscle in lipid planar bilayers (16, 17). In addition, NAADP-induced Ca\(^{2+}\) release from the nuclear envelope of pancreatic acinar cells and from permeabilized pancreatic acinar cells was blocked by RyR antagonists (18, 19).

In human Jurkat T-lymphocytes, we have demonstrated: (i) TCR-CD3-mediated formation of NAADP (9), (ii) Ca\(^{2+}\) signaling induced by microinjection of NAADP in a concentration-dependent fashion (6), (iii) that NAADP-induced Ca\(^{2+}\) signaling consists of both Ca\(^{2+}\) release and Ca\(^{2+}\) entry (6, 20), and (iv) involvement of RyR in both local and global Ca\(^{2+}\) signaling (20, 21). Here we confirm that lysosomes can be functionally destroyed using either hypertonic swelling and physical destruction by glycylyl-phenylalanine 2-naphthylamide (GPN) or inhibition of the lysosomal H\(^{+}\)-ATPase using bafilomycin A1 (11). Although GPN pretreatment resulted in loss of Ca\(^{2+}\)-mobilizing activity of all three second messengers, NAADP, cADPR, and InsP\(_3\), there was no inhibitory effect of bafilomycin A1 on any of the Ca\(^{2+}\)-mobilizing compounds, even when endogenous Ca\(^{2+}\) stores were previously emptied by TCR-CD3 stimulation in the presence of bafilomycin A1. In contrast, emptying the endoplasmic reticular (ER) Ca\(^{2+}\) store by thapsigargin almost completely prevented Ca\(^{2+}\) signaling induced by NAADP. Taken together, our data indicate a major role for the ER, but not for lysosomes, in NAADP-mediated Ca\(^{2+}\) signaling in T cells.
EXPERIMENTAL PROCEDURES

Materials—NAADP, GPN, GdCl₃, and bafilomycin A1 (lot 1) were supplied from Sigma-Aldrich (Deisenhofen, Germany). cADPR was obtained from Biolog (Bremen, Germany). Fura-2/AM, bafilomycin A1 (lots 2 and 3), cathepsin inhibitor 1, and thapsigargin were purchased from Calbiochem. InsP₃ was from Biomol (Hamburg, Germany). The anti-CD3 mouse monoclonal antibody OKT3 was purified from hybridoma supernatant on protein G-Sepharose FF (GE Healthcare, Freiburg, Germany). LysoTracker Red DND-99 was obtained from Molecular Probes (Leiden, The Netherlands).

Cell Culture—Jurkat T-lymphocytes (subclone JMP) were cultured as described previously (22).

Ratiometric Ca²⁺/H₁₀₀₁ Imaging—The cells were loaded with Fura-2/AM as described (22) and kept in the dark at room temperature until use. Thin glass coverslips (0.1 mm) were coated first with bovine serum albumin (5 mg/ml) and subsequently with poly-L-lysine (0.1 mg/ml). Silicon grease was used to seal small chambers consisting of a rubber O-ring on the glass coverslip. Then, 60 µl of buffer A containing 140 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 1 mM Na₂HPO₄, 5.5 mM glucose, and 20 mM HEPES (pH 7.4), and 40 µl of cell suspension (2 × 10⁶ cells/ml) suspended in the same buffer were added into the small chamber (6), and the coverslip was mounted on the stage of a fluorescence microscope (Leica DMIRE2). In some experiments, GdCl₃ (10 or 100 µM) was added to buffer A to block capacitative Ca²⁺/H₁₀₀₁ entry; in these experiments, buffer A without Na₂HPO₄ was used to prevent precipitation of GdPO₄.

Ratiometric Ca²⁺ imaging was performed as described recently (23). We used an Improvision imaging system (Tübingen, Germany) build around the Leica microscope at 100-fold magnification. Illumination at 340 and 380 nm was carried out using a monochromator system (Polychrom IV, TILL Photonics, Gräfelfing, Germany). Images were taken with a gray-scale CCD camera (type C 4742-95-12ER; Hamamatsu, Enfield, United Kingdom) operated in 8-bit mode. The spatial resolution was 512 × 640 pixels at 100-fold magnification. Camera exposure times were 12 ms (at 340 nm) and 4 ms (at 380 nm). The acquisition rate was adjusted to ~14 ratios/min. Raw data images were stored on a hard disk. Confocal Ca²⁺ images were obtained by off-line deconvolution (no-neighbor algorithm) using the volume deconvolution module of the Openlab software as described recently for 3T3 fibroblasts (24). The deconvolved images were used to construct ratio images (340/380 nm) pixel by pixel. Finally, ratio values were converted into Ca²⁺ concentrations by external calibration (23). Data processing was performed using Openlab software, version 3.0.8 or 1.7.8 (Improvision).

Microinjection—Microinjections were carried out as described (25). We used an Eppendorf system (transjector type 5246, micromanipulator type 5171, Eppendorf-Netheler-Hinz, Hamburg, Germany) with Femtotips I and II as pipettes. NAADP, InsP₃, or cADPR were diluted to their final concentration in intracellular buffer (20 mM HEPES, 110 mM KCl, 10 mM NaCl, pH 7.2) and filtered (0.2 µm) before use. Injections were made using the semiautomatic mode of the system with following instrumental settings: injection pressure 60–90 hec-

FIGURE 1. Effect of GPN on LysoTracker (Lyso) staining of lysosomes and on Ca²⁺ release by NAADP, InsP₃, and cADPR in intact Jurkat T-lymphocytes. The effect of GPN on the intensity of LysoTracker staining is shown in A, panels I and II; the cell border is indicated by the dashed line (A, panel II). Jurkat T cells were loaded with LysoTracker Red (75 nM) and incubated with GPN (50 µM) or cathepsin inhibitor 1 (C1–1, 50 µM) plus GPN (10 µM), respectively. Vehicle (Me₂SO) 0.1% v/v and 0.2% v/v) was used as control. B–D, Jurkat T cells were loaded with Fura-2 and subjected to combined Ca²⁺ imaging and microinjection as detailed under “Experimental Procedures.” Time points of the start of incubation and microinjection are indicated by arrows. Cells were incubated with GPN (50 µM) or with Me₂SO (0.1% v/v) as control and microinjected with 100 nM NAADP (B), 4 µM InsP₃ (C) or 100 µM cADPR (D), respectively. Data were synchronized and represent mean values.
FIGURE 2. Effect of bafilomycin A1 (Baf) on LysoTracker staining (Lyso) of lysosomes and on Ca\textsuperscript{2+} release by NAADP in intact Jurkat T-lymphocytes. The effect of bafilomycin A1 on the intensity of LysoTracker staining is shown in A, panels I and II; the cell border is indicated by the dashed line (A, panel II). Jurkat T-lymphocytes were preincubated with different concentrations of bafilomycin A1 and Me\textsubscript{2}SO (DMSO) (0.1% v/v) as control and loaded with LysoTracker Red as described under “Experimental Procedures.” Data are mean ± S.E. (n = 33–84). Three different lots of bafilomycin A1 were used for experiments and were analyzed by RP-HPLC as described under “Experimental Procedures” (B). C–F, Jurkat T cells were loaded with Fura-2, and combined Ca\textsuperscript{2+} imaging and microinjections were carried out as described under “Experimental Procedures.” The time points of microinjections are indicated by arrows. Cells were preincubated with Me\textsubscript{2}SO (0.1%, C) or with different concentrations of bafilomycin A1 and microinjected with 100 nM NAADP. In D, cells were preincubated with 12.5 nM bafilomycin A1 (lot 1); in E, cells were preincubated with 250 nM (lot 3); and in F, cells were preincubated with 1000 nM (lot 3) bafilomycin A1. Combined data are shown in G; data are mean values ± S.E. from the time points 40–65 s (Ca\textsuperscript{2+} peak) or 400 s (Ca\textsuperscript{2+} plateau).
RESULTS

To analyze whether in Jurkat T-lymphocytes lysosomes represent a Ca\(^{2+}\) store sensitive to NAADP, we treated intact cells with GPN, a substrate of cathepsin C. Via osmotic lysis, the metabolic products of GPN selectively disrupt lysosomal membranes (11, 27). The resulting significant loss of lysosome staining by LysoTracker Red was confirmed in Jurkat T cells (Fig. 1A). GPN increased the intracellular Ca\(^{2+}\) concentration by lysing the lysosomes (Fig. 1, B–D, right panels). As described for sea urchin eggs (11), GPN also abolished NAADP-mediated Ca\(^{2+}\) release in T cells (Fig. 1B, right panel). However, Ca\(^{2+}\) release by InsP\(_3\) or cADPR was eliminated, too (Fig. 1, C and D, right panels). Therefore, we used cathepsin inhibitor 1, which is known to reduce the damaging effect of GPN by inhibition of cathepsin B, L, and S and also papain released from the lysosomes. Since cathepsin inhibitor 1 does not inhibit cathepsin C, the disruption of lysosomes by GPN should be unaffected (19). This was indeed confirmed since the intensity of lysosomes labeled with LysoTracker Red after incubation with cathepsin inhibitor 1 (10 \(\mu\)M) plus GPN (50 \(\mu\)M) was reduced similarly as compared with the control (Fig. 1A). However, treatment with cathepsin inhibitor 1 and GPN not only blocked NAADP-mediated Ca\(^{2+}\) release, but also, Ca\(^{2+}\) release by InsP\(_3\) or cADPR was abolished in Jurkat T-lymphocytes (data not shown) as shown above for GPN alone. Thus, although GPN appears to be a powerful tool to destroy lysosomes, the release of lysosomal proteases or other hydrolases seems to interfere with all Ca\(^{2+}\) release systems present in T cells. Since cADPR and InsP\(_3\) are supposed to target the ER, the data obtained with GPN do not allow a clear-cut decision as to whether NAADP solely acts on lysosomes.

As a second approach, we investigated the effect of bafilomycin A1 on global Ca\(^{2+}\) signals induced by NAADP. Bafilomycin A1 is an inhibitor of vacuolar H\(^+\)-pumps powered by ATP (28), prevents organelle acidification, and blocks the Ca\(^{2+}\) reuptake into lysosomes via Ca\(^{2+}\)/H\(^+\)-exchanger since this transport protein requires a proton gradient (11). As LysoTracker Red DND-99 is a weak base and accumulates in acidic compart-

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**FIGURE 3. Effect of bafilomycin A1 (Baf) on Ca\(^{2+}\) release by InsP\(_3\) and cADPR in intact Jurkat T-lymphocytes.**

**A–D,** Jurkat T cells were loaded with Fura-2 and subjected to combined Ca\(^{2+}\) imaging and microinjection as described under “Experimental Procedures.” The time points of microinjections are indicated by arrows. Cells were preincubated with Me\(_2\)SO (0.1% v/v) for 15 min at room temperature. To analyze the effect of bafilomycin A1 on global Ca\(^{2+}\) signals induced by NAADP, we treated intact cells with GPN, a substrate of cathepsin C. Via osmotic lysis, the metabolic products of GPN selectively disrupt lysosomal membranes (11, 27). The resulting significant loss of lysosome staining by LysoTracker Red was confirmed in Jurkat T cells (Fig. 1A). GPN increased the intracellular Ca\(^{2+}\) concentration by lysing the lysosomes (Fig. 1, B–D, right panels). As described for sea urchin eggs (11), GPN also abolished NAADP-mediated Ca\(^{2+}\) release in T cells (Fig. 1B, right panel). However, Ca\(^{2+}\) release by InsP\(_3\) or cADPR was eliminated, too (Fig. 1, C and D, right panels). Therefore, we used cathepsin inhibitor 1, which is known to reduce the damaging effect of GPN by inhibition of cathepsin B, L, and S and also papain released from the lysosomes. Since cathepsin inhibitor 1 does not inhibit cathepsin C, the disruption of lysosomes by GPN should be unaffected (19).

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**Staining of Lysosomes**—Lysosomes were labeled by incubation of cells with 75 nM LysoTracker Red DND-99 for 30 min at room temperature. Cells were added into small chambers (see above), and fluorescence was determined at excitation and emission wavelengths of 575 and 590 nm using the Improvision imaging system as described above.

Then, cells were treated either with 50 \(\mu\)M GPN, 10 \(\mu\)M cathepsin inhibitor 1, plus 50 \(\mu\)M GPN or with Me\(_2\)SO (0.1% v/v) or 0.2% v/v) for 15 min at room temperature. To analyze the effect of bafilomycin A1 on LysoTracker staining, untreated cells were incubated with different concentrations of bafilomycin A1 or 0.1% v/v Me\(_2\)SO as control for 15 min at room temperature, and afterward, lysosomes were labeled with LysoTracker Red DND-99 as described above (26).

**HPLC Analysis of Bafilomycin A1**—RP-HPLC analysis of bafilomycin A1 was performed on a 250 \(\times\) 4.6-mm Multilyp BDS C18-5 \(\mu\) column (CS Chromatographie Service, Langerwehe, Germany) equipped with a 4.0 \(\times\) 3.0-mm guard cartridge containing a C18 (ODS) filter element (Phenomenex, Aschaffenburg, Germany). The separation was performed at a flow rate of 1 ml/min with phosphate buffer (20 mM KH\(_2\)PO\(_4\), pH 6) containing increasing amounts of methanol. The gradi-

topascals, compensatory pressure 30–50 hert used for separation was as follows (number in parentheses represents the percentage of methanol): 0 min (5), 2 min (5), 16 min (100), 20 min (100), 22 min (5), 25 min (5). Bafilomycin A1 was detected using a Diode Array Detector (Agilent, Santa Clara, CA) at 246 nm.
ments, preincubation with bafilomycin A1 concentration-dependently reduced or abolished labeling of lysosomes with LysoTracker Red DND-99 (26), a result that was confirmed in Jurkat T cells (Fig. 2A). For our experiments, we used different lots of bafilomycin A1 purchased from different suppliers. Identity, purity, and content were confirmed by HPLC (Fig. 2B).

Then, cells were preincubated with increasing concentrations of bafilomycin A1 or vehicle (Me2SO 0.1% v/v) and microinjected with 100 nM NAADP (Fig. 2, C–G). No reduction of NAADP-mediated Ca\(^{2+}\)/H\(^{1001}\) signaling was observed after preincubation with bafilomycin A1 at concentrations up to 1 \(\mu\)M (Fig. 2, C–G). In contrast, a slight increase of the NAADP-mediated Ca\(^{2+}\)/H\(^{1001}\) peak at 250 nM bafilomycin A1 was detected as compared with the control (Fig. 2, E and G). Bafilomycin A1 at a concentration that almost completely abolished the lysosome H\(^{+}\) gradient did not affect InSp\(_3\) or cADPR-mediated Ca\(^{2+}\)/H\(^{11001}\) signaling (Fig. 3). Since InSp\(_3\) and cADPR have been shown to release Ca\(^{2+}\)/H\(^{11001}\) from the ER, this control experiment suggests that bafilomycin A1, in contrast to GPN (Fig. 1), in principle can be used to analyze the role of lysosomes in T cell Ca\(^{2+}\)/H\(^{11001}\) signaling.

Lysosomes are supposed to be very tight Ca\(^{2+}\)/H\(^{11001}\) stores, with almost no passive leak of Ca\(^{2+}\)/H\(^{11001}\) ions into the cytosol (11). In Jurkat T-lymphocytes, TCR-CD3 stimulation is known to increase intracellular NAADP levels (9). If lysosomes are the organelles targeted by NAADP, stimulation via the TCR-CD3 complex in the presence of bafilomycin A1 should lead to Ca\(^{2+}\)/H\(^{11001}\) depletion of lysosomal stores. As bafilomycin A1 inhibits the Ca\(^{2+}\)/H\(^{11001}\) reuptake in lysosomes, a second increase of the NAADP level via microinjection should show a reduced or abolished NAADP-mediated Ca\(^{2+}\)/H\(^{11001}\) release.

However, Ca\(^{2+}\)/H\(^{11001}\) signaling by NAADP after preincubation with bafilomycin A1 and subsequent stimulation via TCR-CD3 was not different from untreated controls, indicating no major role for bafilomycin A1-sensitive compartments in NAADP-mediated Ca\(^{2+}\)/H\(^{11001}\) signaling (Fig. 4).

Since no evidence for an involvement for lysosomes in NAADP-induced Ca\(^{2+}\)/H\(^{11001}\) signaling was obtained, the alternative hypothesis (the ER as NAADP-sensitive Ca\(^{2+}\)/H\(^{11001}\) stores) was taken into account. Although the specific inhibitor of sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)/H\(^{11001}\) ATPases (SERCA), thapsigargin (29), is an effective and specific tool to deplete ER/SR-type Ca\(^{2+}\)/H\(^{11001}\) stores, in intact, electrically non-excitable cells, there is often the problem of capacitative Ca\(^{2+}\)/H\(^{11001}\) entry being switched on immediately (30, 31). Thus, to avoid any effects of capacitative Ca\(^{2+}\)/H\(^{11001}\) entry, all further experiments were conducted in the presence of GdCl\(_3\) in the extracellular buffer. Under these conditions, microinjection of 100 nM NAADP resulted in transient elevations of [Ca\(^{2+}\)]\(_i\) with most of the cells returning to basal values within a few hundred seconds (Fig. 5A). Also, the mean amplitude of the initial peak induced by NAADP was signifi-
**DISCUSSION**

Although not confirmed in a great variety of cells, NAADP has been proposed to be an important second messenger in glucose-stimulated pancreatic \( \beta \)-cells (7), in cholecystokinin-mediated \( \text{Ca}^{2+} \) signaling in pancreatic acinar cells (8), and in \( \text{Ca}^{2+} \) signaling in Jurkat T cells stimulated via the TCR-CD3 complex (9). Thus, at least in these selected cell types, the NAADP/\( \text{Ca}^{2+} \) signaling system may play an important role for cellular function. Therefore, important modules of this signaling system are the target organelle and target \( \text{Ca}^{2+} \) channel of NAADP. There has been much debate as to whether NAADP acts on a novel receptor/\( \text{Ca}^{2+} \) channel or whether RyRs respond to NAADP directly or indirectly, e.g., via a separate binding protein (reviewed in Ref. 32). For T cells, we recently demonstrated that functional RyR are required for both local and global \( \text{Ca}^{2+} \) signals induced by NAADP (20, 21). Although RyR are assumed to be localized at the ER (or SR), suggesting that the NAADP-sensitive \( \text{Ca}^{2+} \) pool is located there, convincing data from other cell systems, including sea urchin eggs (10, 11), pancreatic acinar- and \( \beta \)-cells (33), neurosecretory PC12 cells (34), and guinea pig cardiac myocytes (35), prompted us to investigate involvement of a lysosomal \( \text{Ca}^{2+} \) store in NAADP-mediated \( \text{Ca}^{2+} \) signaling in T cells. The first approach, lysis of lysosomes using the cathepsin substrate GPN, has successfully been used in sea urchin eggs (11) and pancreatic acinar cells (33); in sea urchin eggs, GPA-mediated bursting of lysosomes selectively abolished \( \text{Ca}^{2+} \) signaling by NAADP but not by InsP\(_{3}\) or cADPR (11). This result could not be reproduced in T cells; the \( \text{Ca}^{2+} \)-mobilizing effects of all three messengers, NAADP, InsP\(_{3}\), or cADPR, were massively reduced. Since bursting of lysosomes is assumed to release proteases and other hydrolases into the cytosol, it is well imaginable that proteolytic attack of the InsP\(_{3}\) receptor or the RyR is the reason for the lack of effect of InsP\(_{3}\) or cADPR. Thus, although the method may work well in other cell types, such as sea urchin eggs, it appears to be not suitable for T cells.

The second approach, bafilomycin A1, yielded an even better loss of staining of the lysosomes in intact Jurkat T cells (compare Fig. 1A with Fig. 2A), indicating that the \( \text{H}^{+} \) gradient across the lysosomal membrane was effectively destroyed. Lack of lysosomal staining was almost completed at 25 \( \mu \)M bafilomycin A1. However, NAADP-mediated \( \text{Ca}^{2+} \) signaling was not affected by up to 1000 \( \mu \)M bafilomycin A1 in T cells. In contrast, in sea urchin eggs, bafilomycin A1 inhibited \( \text{Ca}^{2+} \) release induced by NAADP in experiments where NAADP was liber-
ated from caged NAADP subsequently two times. Interestingly, the Ca\(^{2+}\) release induced by the first uncaging of NAADP was not influenced, whereas the second one was largely reduced (11). Churchill et al. (11) suggested, “that the lysosomal Ca\(^{2+}\) store is replete and non-leaky in resting cells, and that bafilomycin sensitivity is only revealed once the store is mobilized, making its inhibition use-dependent.” However, in higher eukaryotic cell types, e.g. in pancreatic acinar cells, bafilomycin A1 inhibited cholecystokinin-induced Ca\(^{2+}\) spiking without prior emptying of stores (33). Similarly, glucose-induced Ca\(^{2+}\) signaling in pancreatic \(\beta\)-cells (MIN6 cells) was abrogated by bafilomycin A1 (33). Thus, in higher eukaryotic cells, the NAADP-sensitive Ca\(^{2+}\) stores obviously are not very “non-leaky.” Consequently, the lack of inhibitory effect of bafilomycin A1 on NAADP-induced Ca\(^{2+}\) signals in T cells (Fig. 2) strongly indicates that the NAADP-sensitive Ca\(^{2+}\) store of T cells is not the lysosomal compartment. To be on the safe side, and assuming that T cells are more similar to sea urchin eggs than to pancreatic cells concerning a non-leaky NAADP-sensitive Ca\(^{2+}\) store, a further crucial experiment was performed (Fig. 4); T cells were first stimulated via the TCR-CD3 complex in the presence of bafilomycin A1, and then NAADP was microinjected. TCR-CD3 stimulation elevates NAADP in a biphasic manner (9), and thus, Ca\(^{2+}\) present in NAADP-sensitive stores was released. Due to the presence of bafilomycin A1, any reuptake of Ca\(^{2+}\) into acidic stores was inhibited. Nevertheless, there was no inhibition of subsequent Ca\(^{2+}\) signaling induced by microinjection of NAADP, ruling out the possibility that T cells possess a very tight (= non-leaky) NAADP-sensitive lysosomal Ca\(^{2+}\) store.

In contrast to the idea that pancreatic acinar cells exclusively possess a lysosomal Ca\(^{2+}\) store sensitive to NAADP, Gerasimenko et al.
obtained evidence for NAADP-mediated Ca\(^{2+}\) release from the nuclear envelope (18) and partially from both the ER and acidic organelles (19). In a similar approach in which the activation of capacitative Ca\(^{2+}\) entry was blocked by GdCl\(_3\), we emptied ER Ca\(^{2+}\) stores by the addition of thapsigargin. Since subsequent microinjection of NAADP did not induce Ca\(^{2+}\) release in most of the cells investigated, it is very likely that NAADP indeed acts on an endoplasmic reticular Ca\(^{2+}\) store in T cells. In contrast, in the complimentary experiment (prior microinjection of NAADP followed by the addition of thapsigargin), the effect of thapsigargin was reduced as compared with prior microinjection of intracellular buffer (Fig. 6). These data indicate that indeed the filling state of the ER was decreased by approximately 35% by prior injection of NAADP. A similar experiment carried out using GPN instead of thapsigargin to analyze the filling state of the lysosomes resulted in no difference regardless of whether the cells had been microinjected by NAADP or intracellular buffer. Taken together, our results demonstrate a major role for the ER, but not for lysosomes, in NAADP-mediated Ca\(^{2+}\) signaling in T cells.

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