NAADP-mediated Ca\(^{2+}\) signaling via type 1 ryanodine receptor in T cells revealed by a synthetic NAADP antagonist

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The nucleotide NAADP was recently discovered as a second messenger involved in the initiation and propagation of Ca\(^{2+}\) signaling in lymphoma T cells; but its impact on primary T cell function is still unknown. An optimized, synthetic, small molecule inhibitor of NAADP action, termed BZ194, was designed and synthesized. BZ194 neither interfered with Ca\(^{2+}\) mobilization by D-\(\alpha\)-myo-inositol 1,4,5-trisphosphate or cyclic ADP-ribose nor with capacitative Ca\(^{2+}\) entry. BZ194 specifically and effectively blocked NAADP-stimulated \(^{3}H\)ryanodine binding to the purified type 1 ryanodine receptor. Further, in intact T cells, Ca\(^{2+}\) mobilization evoked by NAADP or by formation of the immunological synapse between primary effector T cells and astrocytes was inhibited by BZ194. Downstream events of Ca\(^{2+}\) mobilization, such as nuclear translocation of “nuclear factor of activated T cells” (NFAT), T cell receptor-driven interleukin-2 production, and proliferation in antigen-experienced CD4\(^+\) effector T cells, were attenuated by the NAADP antagonist. Taken together, specific inhibition of the NAADP signaling pathway constitutes a way to specifically and effectively modulate T-cell activation and has potential in the therapy of autoimmune diseases.

antagonism | nucleotide | second messenger | synthesis

Ca\(^{2+}\) signaling is one of the essential signal transduction systems involved in T-cell activation. Upon formation of immunological synapses, a complex network of Ca\(^{2+}\) signaling modules is activated in a spatiotemporal fashion. One of the major mechanisms involved, capacitative Ca\(^{2+}\) entry via Orai1/CRACM1 (1–4), requires continuous Ca\(^{2+}\) release from intracellular pools for activation. Besides Ca\(^{2+}\) release by D-\(\alpha\)-myo-inositol 1,4,5-trisphosphate (InsP\(_3\), ref. 5), we have shown that two additional messengers, cyclic ADP-ribose (cADPR; ref. 6) and NAADP (Fig. 1\(^{1}\)), indicate that nicotinic acid derivatives might be suitable for chemical biological intervention as NAADP antagonists and for possible drug design. Since NAADP is highly active in Ca\(^{2+}\) release, while the structurally related parent compound NADP (Fig. 1\(^{4}\)) is inactive (11), it is very likely to be the nicotinic acid moiety that interferes with NAADP-mediated Ca\(^{2+}\) signaling. Indeed, since cellular uptake of the polar nicotinic acid is slow and


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Fig. 1. Nicotinic acid and its derivative BZ194 inhibit NAADP-mediated Ca\(^{2+}\) signaling in T cells. (A) Structures of NAADP and NADP. (B) NAADP-induced Ca\(^{2+}\) signaling in T cells is inhibited by nicotinic acid. Jurkat T cells were loaded with Fura2-AM and were microinjected with 100 nM NAADP, 100 nM NAADP plus 1 mM nicotinamide, or 100 nM NAADP plus 1 mM nicotinic acid, or intracellular buffer. Free cytosolic Ca\(^{2+}\) (\([\text{Ca}^{2+}]_{i}\) ) was measured. Mean data of 4–12 experiments are shown (Upper). The bar chart (Lower) summarizes the data (Ca\(^{2+}\) peak and Ca\(^{2+}\) plateau; means ± SEM, n = 4–12; *, P ≤ 0.05; ns, not significant). (C) Inhibition of NAADP-induced Ca\(^{2+}\) signaling upon co-injection with BZ194. Jurkat T cells were co-injected with 100 nM NAADP and increasing concentrations of BZ194. Mean data of 5–13 experiments are shown. (D) Concentration-response curve of NAADP-induced Ca\(^{2+}\) signaling upon microinjection with BZ194 (mean ± SEM, n = 5–13; *, P ≤ 0.05). (E) BZ194 does not directly interfere with InsP\(_3\)- or cADPR-mediated Ca\(^{2+}\) release. Jurkat T cells were loaded with Fura2/AM. Thereafter, single cells were co-injected with 1 mM BZ194 and either NAADP (100 nM), InsP\(_3\) (4 μM), or cADPR (100 μM), and Ca\(^{2+}\) signaling was recorded. Relative values are shown as percentages of NAADP-induced [Ca\(^{2+}\)]\(_i\). Data represent means ± SEM, n = 5–13; *, P ≤ 0.05; ns, not significant.

Antagonism of Ca\(^{2+}\) Signaling in Primary T Cells by NAADP Antagonist BZ194. Having demonstrated specific antagonism of NAADP effects at the purified RyR1 and in the model T cell line Jurkat, primary myelin-basic-protein-reactive rat T cells (TMBP cells) were used to study the role of NAADP activation in effector T cells. TMBP cells express both RyR1 and RyR3, although RyR3 to a lesser extent (Fig. 3A). RyR1 showed a significant co-localization with an ER marker, while RyR2 was not detected (Fig. 3A).

BZ194-type compounds with varying lengths and types of side chains R1 and/or R2 (Fig. S1A), were tested with intact TMBP cells using antigen-induced proliferation as read-out (Fig. S1B). Using iterative exploration of substituents the single octyl side chain of BZ194 proved to be most suitable for membrane permeability and neither a lack of side chain in CMA008 had any effect on TMBP cell signaling of TMBP cells evoked by anti-CD3 signals. While presentation of MBP by the astrocytes BZ194 blocked Ca\(^{2+}\) signaling in intact TMBP cells upon stimulation with the specific antigen. Using astrocytes pulsed with MBP, Ca\(^{2+}\) signaling was recorded upon formation of the immunological synapse (Fig. 3B). While presentation of MBP by the astrocytes resulted in substantial Ca\(^{2+}\) signaling, reaching a plateau phase within about 4 min, BZ194 significantly diminished this response (Fig. 3 B and C). Analysis on a single-cell level revealed that treatment with BZ194 significantly decreased the percentage of T cells showing high Ca\(^{2+}\) responses. With MBP as antigenic protein, 21 out of 30 cells showed Ca\(^{2+}\) signals >200 nM, whereas upon BZ194 preincubation Ca\(^{2+}\) signals >200 nM were observed in only 17 out of 31 cells. If only Ca\(^{2+}\) signals >300 nM were taken into account, with MBP as antigenic protein, 19 out of 30 cells responded, while BZ194 decreased the number of responding cells to 10 out of 31. Ca\(^{2+}\) signaling of TMBP cells evoked by anti-CD3

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monoclonal antibody, and subsequent crosslinking of the primary antibody was also sensitive to BZ194. Both the initial Ca\(^{2+}\) peak and the sustained Ca\(^{2+}\) plateau were reduced in a concentration-dependent fashion with the plateau being more sensitive compared with the initial peak (Fig. 3 D and E). The antagonistic effect of BZ194 required preincubation periods (Fig. 3F), indicating moderate membrane permeability and/or activation of drug export systems.

Capacitative Ca\(^{2+}\) entry was not inhibited by BZ194 since Ca\(^{2+}\) signaling induced by thapsigargin in TMBP cells was unaffected (Fig. 3 G and H). Taken together, our data suggest a crucial role for NAADP as trigger of Ca\(^{2+}\) signals during the process of immunological synapse formation.

**NAADP Antagonist BZ194 Suppressed Downstream Events of Ca\(^{2+}\) Signaling.** Calcium signaling in T cells activates Ca\(^{2+}\)/calmodulin dependent phosphatase calcineurin leading to "nuclear factor of T cells" (NFAT) dephosphorylation and translocation into the nucleus. BZ194 significantly reduced nuclear translocation of NFAT following combined stimulation of rat TMBP cells with anti-CD3 and anti-CD28 antibodies (Fig. 4 A and B).

Furthermore, BZ194 efficiently suppressed antigen-induced production of the major T cell cytokine IL-2, as determined on the mRNA and protein levels (Fig. 4 C and D). Importantly, BZ194-mediated regulation of IL-2 levels was reversible. Re-stimulation of the suppressed T cells in the absence of BZ194 led to complete reconstitution of the original cytokine profile (Fig. S3). In a similar fashion as for rat T cells, in human MBP-specific T-cell clones a decrease in IL-2 mRNA, IL-2 production and proliferation was observed upon preincubation with BZ194 (Fig. S4).

The NAADP antagonist suppressed antigen-induced T-cell proliferation at concentrations higher than 50 \(\mu M\). As expected, the highly polar and membrane impermeable parent compound nicotinic acid was ineffective (Fig. S1B). Non-stimulated T cells tolerated prolonged treatment (48 h) with BZ194; furthermore, T cells which had previously been suppressed by BZ194 remained responsive to their antigen, when re-challenged with antigen-pulsed, antigen-presenting cells (APCs) after wash-out of BZ194 (Fig. S5). Moreover, BZ194 treatment 48 h after stimulation did not impair T-cell proliferation and did not induce increased cell death (Fig. S5). Together with the microinjection (Fig. 1 B–E) and the \(^{3}H\)ryanodine binding results (Fig. 2), these data indicate very specific targeting of RyR1 since (i) wash-out of BZ194 reverted the inhibition, and (ii) late stages of T-cell activation (>48h) were unaffected by addition of BZ194. This interpretation was further substantiated by directly demonstrating that BZ194 did not affect cellular signaling cascades beyond Ca\(^{2+}\) signaling. Bypassing early Ca\(^{2+}\) signaling by stimulating TMBP cells with Ca\(^{2+}\)-ionophore ionomycin either in combination with anti-CD3/anti-CD28 antibodies or with phosphor ester (PMA) rendered the T cells non-susceptible to BZ194 inhibition (Fig. S4E). Similar inhibition of proliferation by BZ194 treatment was observed in ovalbumin and S100\(\alpha\)-specific rat T cells (Fig. S6).

**Discussion**

Recent reports on the role of Ca\(^{2+}\) signaling in activation of T cells indicate a complex network involving NAADP as initial Ca\(^{2+}\) trigger (7–10, reviewed in ref. 17). Here we report that the nicotinic acid residue of NAADP is suitable as a starting point for the development of NAADP antagonists. In sea urchin eggs, the 2'-phospho-group of the ribose bound to the adenine-linked ribose, the 6-NH\(_{2}\) group of adenine and the nicotinic acid moiety were identified as important structural elements for Ca\(^{2+}\) release (33). Since these 3 structural moieties are distributed over the whole NAADP molecule, it was deemed very difficult to design a potential antagonist molecule comprising alterations in all 3 structural elements. Thus, we started with simple N-alkylated analogues of nicotinic acid to which lipophilic groups to enhance membrane permeability were covalently bound. Additionally, we explored other substitutions around the aromatic nucleus to optimize activity. A range of potential ligands was synthesized to gain the optimal balance between cellular permeability, activity, and physicochemical properties. In structure-activity studies, we found that little variation beyond a moderate-sized hydrophobic side chain was tolerated by T cells. In fact, the 8 carbon-side chain analog BZ194 was effective with an IC\(_{50}\) around 100 \(\mu M\). The extra hydrophobicity in BZ194 can also be distributed over 2 substituents on nitrogen atoms giving a similar effect in BZ52 and thus is consistent without further optimization. In contrast to BZ194 and BZ52, BZ23, an analog with a substituted aromatic side chain, enhanced proliferation of TMBP cells. The molecular mechanism underlying this interesting observation will be the subject of further experiments.

One obvious concern in using pharmacological compounds is the question of their specificity. To this end, we excluded that BZ194 affects the other known Ca\(^{2+}\)-mobilizing pathways, that is InsP\(_{3}\)-or cADPR-dependent Ca\(^{2+}\) release, or capacitative Ca\(^{2+}\) entry. Furthermore, (i) bypassing inhibition of NAADP signaling by Ca\(^{2+}\)-ionophore, (ii) reversibility of inhibition of proliferation upon wash-out of BZ194, and (iii) lack of effect of BZ194 addition at later time points of T-cell activation altogether strongly suggest high specificity of the drug used. Finally, we also ensured that BZ194 does not block physiological Ca\(^{2+}\) signaling via different mechanisms in other cell types, for example, in electrically paced mouse cardiac myocytes or human HeLa cells stimulated via G protein coupled muscarinic acetylcholine receptors.
Recently, 1-carbamoylmethyl-3-carboxy pyridinium iodide (abbreviated CMA008; see Fig. S1 A and B) was shown to antagonize NAADP effects in sea urchin eggs and murine pancreatic acinar cells (34). Although this analog is structurally related to BZ194, no inhibitory activity in T cells was observed. This non-sensitivity of T cells vs. sensitivity of pancreatic acinar cells reflects the different nature of the target Ca²⁺ store and the NAADP receptor involved. While a novel NAADP-sensitive channel has been proposed for those cell types with acidic stores sensitive to NAADP (20–25), strong evidence for RyR1 as NAADP target was obtained in T cells since NAADP modulated high-affinity [³H]ryanodine binding. This in turn reflects RyR1 channel opening, as the radioligand binds to the open conformation of the ion channel (32, 35–37). Importantly, the BZ194 concentrations needed to suppress NAADP induced Ca²⁺ release in Jurkat T lymphocytes (Fig. 1D) equal that needed to prevent NAADP stimulated [³H]ryanodine binding (Fig. 2B). Thus, these data suggest that the RyR1 is the primary target for BZ194. RyR3 is expressed to a lesser extent in effector T cells. Gene silencing experiments in Jurkat T cells indicate that RyR3 is targeted by cADPR (38). However, defining the precise interaction between cADPR and any RyR subtype, as shown in Fig. 2 of this report for NAADP and highly purified RyR1, requires further extensive experiments. Alternatively proposed NAADP targets, such as TRP-ML 1 (24, 25) that are localized to lysosomes, do not appear to play a major role in T cells since the ER rather than lysosomes are involved in NAADP mediated Ca²⁺ signaling in T cells (31).

The present study complements published models on temporal events of Ca²⁺ release. We show here that NAADP via RyR1 activation acts as the initializing trigger, which controls further Ca²⁺ release events by supplying local Ca²⁺ signals for Ca²⁺ (39) or cADPR-induced Ca²⁺ release. NAADP mediated Ca²⁺ release starts rapidly within a few seconds upon TCR/CD3 ligation (8). By contrast, InsP₃- and cADPR-induced Ca²⁺ release, mediated releases appear within a few minutes (39) or within tens of minutes, respectively (6). A possible reason for this complex regulatory Ca²⁺ release network may be the need for temporal checkpoints, at which the T cell, based upon the input via the TCR/CD3 complex and diverse co-receptors, can control whether to proceed with activation or not (40). The decision whether to continue or to stop Ca²⁺ signaling may ultimately result in cell proliferation and exertion of effector functions, or it may drive the T cell into a state of unresponsiveness or cell death. The NAADP/ Ca²⁺ signaling pathway seems to control this decision, since expression of the major cytokine involved in T cell proliferation, IL-2, depends on a full, NAADP-initiated Ca²⁺ response. If the NAADP pathway is blocked by specific inhibitors, Ca²⁺ signaling is only partially activated. Therefore, it does not provide a sufficient long-lasting increase in the free cytosolic Ca²⁺ concentration to allow Ca²⁺/calmodulin- and calcineurin-dependent translocation of NFAT into the nucleus (41).
chemistry of BZ194-treated or control (DMSO-treated) TMBP cells 0, 30, or 60 min and human TMBP cells. (Ca\textsuperscript{2+}; or isotype control staining. (Scale bars, 10

least of measurements from 5 independent experiments. (Fig. 2 B and D) supports this assumption. In terms of potential clinical usage such a mechanism of action may result in less effects on the basal RyR1 activity, but increased inhibition if the RyR1 is activated. Thus, one may assume that side effects by such a mechanism of action could be reduced compared with a state-independent inhibitor of the RyR1.

Therefore, the involvement of the NAADP/Ca\textsuperscript{2+} signaling pathway in a primary T-cell model relevant for the pathogenic context of T cell-mediated autoimmune has been demonstrated in vitro using a purpose-designed and optimized synthetic small molecule antagonist. The use of such NAADP antagonists in the corresponding animal model of experimental autoimmune encephalomyelitis (EAE) is the subject of ongoing investigations. Treatment with BZ194 in vivo was found to significantly ameliorate clinical disease. Thus, this approach may result in additional therapeutic agents for treating autoimmune diseases such as multiple sclerosis.

Methods
Materials. NAADP was supplied by Sigma. cADPR and InsP\textsubscript{3} were obtained from Biolog. All reagents and solvents were of commercial quality and were used directly unless otherwise described.

Synthesis of 3-Carboxy-1-octylcarbamoylmethylpyridinium Bromide (BZ194). For synthesis, nicotinic acid (1.62 mmol) and 2-bromo-N-octylacetamide (1.62 mmol) were dissolved in dry dimethylformamide (DMF, 4 mL) and the reaction solution was heated at 65 °C overnight in the dark. DMF was evaporated in vacuo and the resulting residue was dissolved in a small amount of methanol.

The crude compound was precipitated by dropwise addition of ether. 3-Carboxy-1-octylcarbamoylmethylpyridinium was further purified by flash chromatography (0–10% methanol against dichlormethane) and was crystallized in methanol and acetone. The compound exhibited spectroscopic and analytical properties commensurate with its structure using standard techniques as detailed in the SI Methods.

High-Affinity \textsuperscript{3}H\textsuperscript{H}yanodine Binding. RyR1 was purified from rabbit skeletal muscle (42). Purity of the RyR1 preparation was controlled by SDS/PAGE and silver staining (34). High-affinity \textsuperscript{3}H\textsuperscript{H}yanodine binding was carried out with 7.5–20 \textmu g purified RyR1, which was incubated for the indicated times at 30 °C in a buffer containing 20 mM Heps (pH 7.4), 140 mM KCl, 50 mM NaCl, 2.5% phosphatidyl choline, 7 mM CHAPS, and 20 mM \textsuperscript{3}H\textsuperscript{H}yanodine supplemented by protease inhibitors (1 \textmu M leupeptin, 1 \textmu M aprotenin, and 100 \textmu M Pefablok). The free calcium concentration was adjusted to 6 \textmu M by ratio of CaCl\textsubscript{2} and EGTA. The reactions were terminated by filtration. Specific binding was determined by subtraction of nonspecific binding in the presence of 20 \textmu M cyanodine (42).

Generation and Culturing of T Cells. Jurkat lymphoma T cells (clone JUMP) were cultured as described previously (43). Rat antigen-specific T cell clones were obtained from lymph node preparations of immunized Lewis rats. Stimulation, expansion, and culture of specific rat T cells were conducted under conditions as described (44). Details concerning generation of MBP-specific CD4\textsuperscript{+} T cells (TMBP) retrovirally engineered to express the marker gene EGFP are provided in the SI Methods.

Cytosolic Ca\textsuperscript{2+} Measurements. Both Jurkat T lymphoma and rat TMBP cells were loaded with Fura-2/AM (Calbiochem) as described (44) and kept in the dark at room temperature until use. Intracellular Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]) was determined in suspensions of Fura-2-loaded rat TMBP cells which had been preincubated with BZ194 or DMSO at the indicated concentrations for at least 8 h in a Hitachi F-2000 fluorometer as described previously (46). More detailed information about the ratiometric Ca\textsuperscript{2+} measuring method is provided in the SI Methods.

Microinjections. Intracellular injections were carried out as described previously (7, 9, 10, 31). More detailed information is provided in the SI Methods.
Methods

detailed information about proliferation assays in rat T cells is provided in the evaluate proliferation. Radioactivity was determined as described (47). More

Quantitative PCR and ELISAs. Detailed information is provided in the SI Methods.

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