NAADP signaling: Master manipulation

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Nicotinic acid adenine dinucleotide phosphate (NAADP) serves as a pivotal Ca^{2+} mobilizing second messenger. New work by Krukenberg et al [1] introduces MASTER-NAADP, a membrane-permeable, stabilized, and bio-reversibly protected precursor of NAADP, as a novel tool to manipulate NAADP signaling in live cells.

The Ca^{2+} mobilizing activity of NAADP was discovered in sea urchin egg homogenates [2]. In most cells, it evokes Ca^{2+} release through endo-lysosomal two-pore channels (TPCs) which in turn triggers secondary Ca^{2+} release from the endoplasmic reticulum [3]. Activation of target channels by NAADP, which also includes ryanodine receptors (RyR), is indirect requiring NAADP-binding proteins. These have been identified in recent years as Jupiter microtubule associated homolog 2 (JPT2) and like-Sm protein 12 (LSM12) [4]. NAADP, is a charged molecule at physiological pH and thus cell-impermeant. This necessitates laborious microinjection procedures for delivery to intracellular receptors. A cell-permeable small molecule that functionally mimics the effects of NAADP (TPC2-A1-N) was recently identified [5]. However, it bypasses NAADP-binding proteins and likely acts directly on TPC2 [6]. Thus, the demand for selective and potent cell-permeable NAADP analogues to further NAADP research remains high.

The new work presents the development and evaluation of MASTER-NAADP, which releases an NAADP analogue upon esterase-mediated cleavage of the protective groups (Fig. 1a). Its synthesis is a tour de force. It was obtained by coupling a protected benzoic acid c-nucleotide with a selectively protected 3'-fluoroadenosine-2',5'-bisphosphate in good yield (~60%). These two building blocks required separate syntheses. The 'northern' benzoic acid derivative was made in 6 steps (~6% yield) and the 'southern' AMP derivative was made in 11-steps (~2% yield). Krukenberg et al also synthesized a MASTER version of NADP, which does not release Ca^{2+}, for control experiments through an equally complex process. Overall, these multi-step convergent syntheses producing milligram quantities of two novel, stable, and completely characterized compounds suitable for testing in biological systems are significant achievements.

It should be stressed that the deprotected MASTER compounds i.e. those expected to be released in cells differ from NAADP and NADP. Both deMASTER-NAADP and deMASTER-NADP incorporate c-nucleotides whereas the endogenous counterparts are pyridine dinucleotides. This is potentially advantageous, as it would render the products resistant to metabolism by NADase-like enzymes.

Additionally, deMASTER-NAADP is fluorinated at the 3' position of adenosine and unfluorinated deMASTER-NADP is a mixture of 2' and 3' phosphorylation products. Thus, MASTER-NADP is not a perfect control for MASTER-NAADP. Moreover, previous structure-activity relationships for NAADP revealed the importance of the 3' position of adenosine. NAADP phosphorylated at the 3' (3'NAADP) instead of the 2' (NAADP) position or at both positions (2',3' cyclic-NAADP) were much less potent in releasing Ca^{2+} than NAADP [7]. This raises the possibility that the activity of deMASTER-NAADP might be lower relative to NAADP due to fluorination.

The authors tested deMASTER-NAADP *in vitro*. In permeabilized Jurkat T lymphocytes, it induced Ca^{2+} release from internal stores. In contrast, deMASTER-NADP did not. They also compared activity with NAADP at a single concentration. Reports of NAADP action in broken preparations outside of the sea urchin egg are scant and likely reflect loss of soluble NAADP binding proteins required for NAADP action [4]. Consistent with this, Ca^{2+} release evoked by NAADP and deMASTER-NAADP was only observed upon exogenous addition of JPT2. Curiously, Ca^{2+} release required the medium Ca^{2+} concentration corresponding to the cytosol to be elevated to ~600 nM. How Ca^{2+} was set and how the evoked Ca^{2+} elevations (20-40 nM) were reliably detected above this background was unclear.

The authors went on to demonstrate that MASTER-NAADP evoked localized Ca^{2+} signals that propagated into global Ca^{2+} signals across various intact cell types, including Jurkat-T lymphocytes, primary T lymphocytes, KHYG-1 Natural Killer cells, and Neuro2A cells. The control compound, MASTER-NADP, had less effect. This was most striking in Neuro2A cells where nearly all cells responded to 100 μM MASTER-NAADP whereas almost none responded to MASTER-NADP. However, Ca^{2+} responses varied substantially between cell types. In Jurkat T lymphocytes, the responses were essentially non-specific because both MASTER compounds at 100 μM evoked similar responses. Specificity was only demonstrable at much lower concentrations when cell responsiveness (~30% v ~10% for MASTER-NAADP and MASTER-NADP, respectively). As aptly stated by the authors *'in T cells such high concentrations of the MASTER compounds are neither necessary nor useful to study NAADP signaling'*. This brings us back the reliability of MASTER-NADP as a control as differences in pharmacokinetics or deprotection relative to MASTER-NAADP might be relevant when cell responsiveness between the two is only modestly different. Importantly, the study again confirmed that MASTER-NAADP required JPT2 for Ca^{2+} release, as knockout cells lacking the protein were less responsive.

The authors also showed that deMASTER-NAADP but not deMASTER-NADP stimulated the activity of purified RyR1 channels in lipid bilayers in single channel recordings using K^+ as the charge carrier. These results are consistent with an early single channel study reporting similar activation by NAADP [8]. But as in permeabilized Jurkat T lymphocytes, the effect of deMASTER-NAADP required recombinant JPT2. This contrasts to the original bilayer study [8] performed well before the discovery of JPT2 and thus in its presumed absence. The negative results in recording lacking JPT2 reported here marry with more contemporary findings reporting lack of effect of NAADP on RyR1 [9]. Ryanodine receptors are activated by cytosolic Ca^{2+} and in accord, recordings were performed in its presence (1 μM). Indeed, the Ca^{2+} dependence of NAADP action in permeabilized Jurkat T lymphocytes, coupled with increased basal Ca^{2+} levels in exemplar Ca^{2+} imaging records of intact cells responsive to MASTER-NAADP relative to cells unresponsive to MASTER-NADP, appears to suggest that NAADP requires Ca^{2+} for activity. This contrasts findings in sea urchin egg homogenates [10] and points to RyR1 as an amplifier of NAADP-mediated Ca^{2+} signals rather than a trigger.

Overall, MASTER-NAADP is a welcome addition to the arsenal of tools for manipulating NAADP-evoked Ca^{2+} signals. Future work would ideally simply its synthesis, and define the properties of the de-protected NAADP derivative to help understand the intriguing variability in efficacy between cells and thereby maximize the impact of this exciting new probe.

[1] S. Krukenberg, F. Möckl, M. Weiß, P. Dekiert, M. Hofmann, F. Gerlach, K.J. Winterberg, D. Kovacevic, I. Khansahib, B. Troost, M. Hinrichs, V. Granato, M. Nawrocki, T. Hub, V. Tsvilovskyy, R. Medert, L.-M. Woelk, F. Förster, H. Li, R. Werner, M. Altfeld, S. Huber, O.B. Clarke, M. Freichel, B.-P. Diercks, C. Meier, A.H. Guse, MASTER-NAADP: a membrane permeable precursor of the Ca2+ mobilizing second messenger NAADP, Nat Commun, 15 (2024) 8008.
[2] H.C. Lee, R. Aarhus, A Derivative of NADP Mobilizes Calcium Stores Insensitive to Inositol Trisphosphate and Cyclic ADP-ribose (*), Journal of Biological Chemistry, 270 (1995) 2152-2157.
[3] Y. Yuan, V. Arige, R. Saito, Q. Mu, G.C. Brailoiu, G.J.S. Pereira, S.R. Bolsover, M. Keller, F. Bracher, C. Grimm, E. Brailoiu, J.S. Marchant, D.I. Yule, S. Patel, Two-pore channel-2 and inositol trisphosphate receptors coordinate Ca^{2+} signals between lysosomes and the endoplasmic reticulum, Cell Reports, 43 (2024).
[4] S. Patel, M. Gregori, Y. Yuan, Teaming with NAADP, Science Signaling, 14 (2021) eabh2798.
[5] S. Gerndt, C.-C. Chen, Y.-K. Chao, Y. Yuan, S. Burgstaller, A. Scotto Rosato, E. Krogsaeter, N. Urban, K. Jacob, O.N.P. Nguyen, M.T. Miller, M. Keller, A.M. Vollmar, T. Gudermann, S. Zierler, J. Schredelseker, M. Schaefer, M. Biel, R. Malli, C. Wahl-Schott, F. Bracher, S. Patel, C. Grimm, Agonist-mediated switching of ion selectivity in TPC2 differentially promotes lysosomal function, Elife, 9 (2020) e54712.
[6] R. Saito, Q. Mu, Y. Yuan, M. Rubio-Alarcón, M. Eznarriaga, P. Zhao, G. Gunaratne, S. Kumar, M. Keller, F. Bracher, C. Grimm, E. Brailoiu, J.S. Marchant, T. Rahman, S. Patel, Convergent activation of Ca^{2+} permeability in two-pore channel 2 through distinct molecular routes, Science Signaling, 16 (2023) eadg0661.
[7] H.C. Lee, R. Aarhus, Structural Determinants of Nicotinic Acid Adenine Dinucleotide Phosphate Important for Its Calcium-mobilizing Activity*, Journal of Biological Chemistry, 272 (1997) 20378-20383.
[8] M. Hohenegger, J. Suko, R. Gscheidlinger, H. Drobny, A. Zidar, Nicotinic acid–adenine dinucleotide phosphate activates the skeletal muscle ryanodine receptor, Biochemical Journal, 367 (2002) 423-431.
[9] L.E. Wagner, L.A. Groom, R.T. Dirksen, D.I. Yule, Characterization of ryanodine receptor type 1 single channel activity using “on-nucleus” patch clamp, Cell Calcium, 56 (2014) 96-107.
[10] E.N. Chini, T.P. Dousa, Nicotinate-adenine dinucleotide phosphate-induced Ca2+-release does not behave as a Ca2+-induced Ca2+-release system, Biochemical Journal, 316 (1996) 709-711.

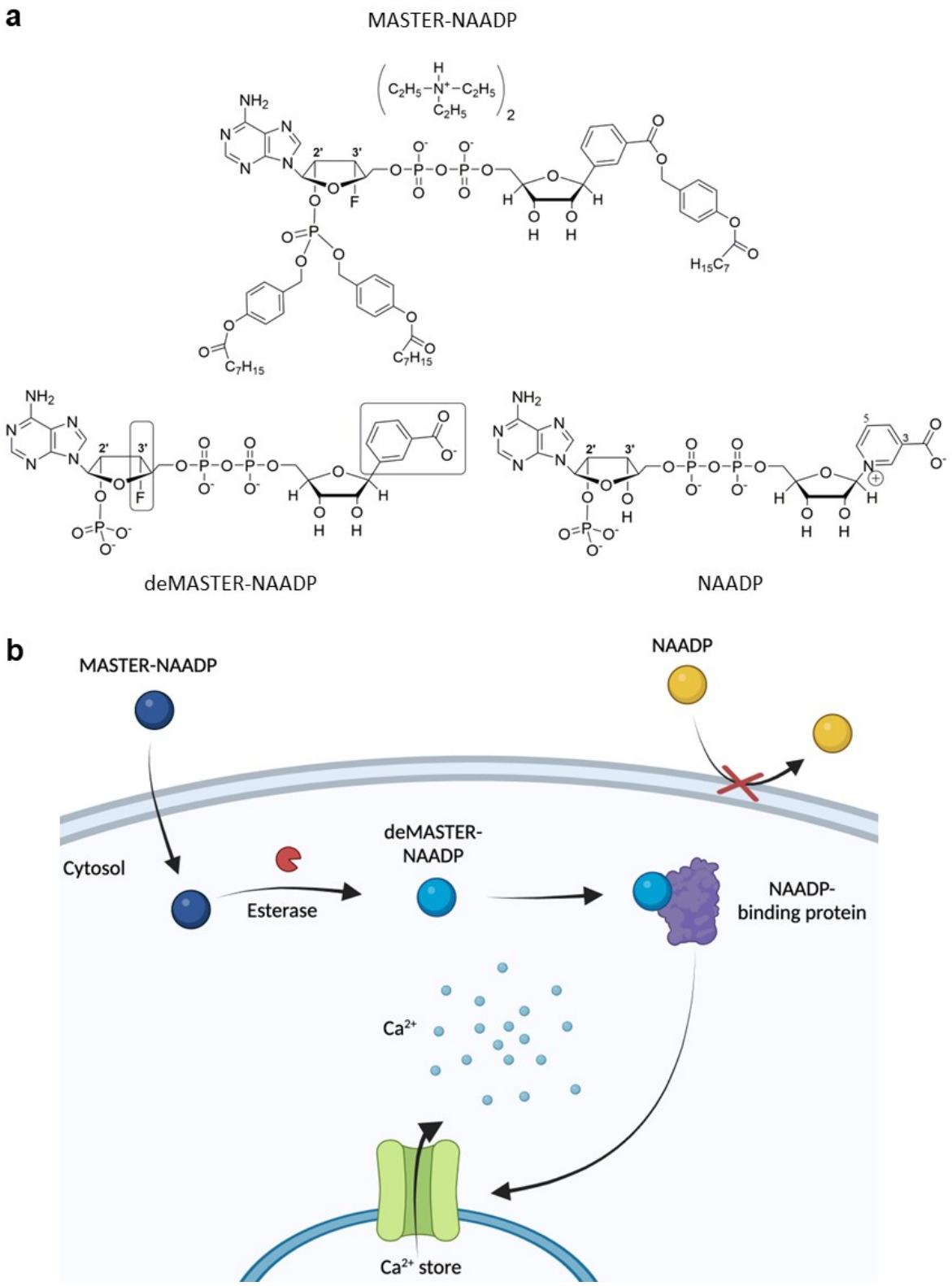

Figure 1. MASTER-NAADP. a, Chemical structures of MASTER-NAADP, its deprotected derivative (deMASTER-NAADP) and NAADP. Structural differences between deMASTER-NAADP and NAADP are highlighted by the rectangles. **b**, Schematic depicting diffusion of MASTER-NAADP (but not NAADP) into cells, its cleavage by endogenous esterases to generate deMASTER-NAADP and activation of Ca^{2+} release through binding to NAADP-binding proteins.