

METHODS

Reconstitute the lysosome into lipid bilayer and characterize the NAADP-sensitive Ca²⁺ release channel. The purified lysosomal membranes from human fibroblast cells were reconstituted into planar lipid bilayers with a method previously described by Li, et al. (Li PL, et al. *Am J Physiol Heart Circ Physiol*. 2002 Apr;282(4):H1304-10). Axopatch 200B amplifier was applied for the bilayer potential control and currents recording. The channel open probability (NP_o) in the lipid bilayer was determined from 3 to 5 min recordings and all lipid bilayer experiments were performed at room temperature (~20° C).

NAADP delivery and Ca²⁺ ratiometric measurement. Ultrasound (Rich-Mar Sonitron 2000) and Optison (Perflutren protein type A microspheres) were employed to deliver NAADP into human fibroblast cells. Fura 2 was used in the fluorescence image analysis to determine the Ca²⁺ release effects by NAADP with the methods detailed in our publication (Zhang F, Li PL, et al. *Am J Physiol Heart Circ Physiol*. 2006 Jul;291(1):H274-82).

Confocal microscopic analysis of endosomes and lysosomes dynamic interaction. Human fibroblast cells were incubated with Dextran-conjugated Rhodamine (0.4 mg/ml) for 4 hrs in color free M199 cell culture medium containing 10% FBS at 37°C, 5% CO₂, followed by 20 h chase in Rhodamine free medium. These Rhodamine loaded fibroblasts were subsequently incubated with Oregon green 488 (0.5 mg/ml) for 10 min then treated with PPADS (100 nM) or bafilomycin A1 (100 nM) and chased for another 10 min, and then viewed by an Olympus Fluoview System, which consists of an Olympus BX61WI inverted microscope with an Olympus Lumplan F1×60, 0.9 numerical aperture, and water-immersion objective. For NAADP-treated groups, immediately before transferring to confocal microscopic stage, NAADP was delivered to the cells at a concentration of 100 nM by our published microbubble method. The yellow spot intensity in the merged image of lysosomes (Rhodamine Red) and endosomes (Oregon green), which resulted from the dynamic interaction of lysosomes and endosomes, were quantified by Image Pro-Plus software and normalized to the lysosomes intensity.

Confocal microscopic determination of BODIPY-LacCer green metabolism rate. Lysosomes in Human fibroblasts were labeled by incubation of cells with 75 nM LysoTracker Red DND-99 in color free M199 medium for 30 min, then washed out. The lysosomes stained fibroblast cells were then incubated in cell culture medium containing 5 μM BODIPY-FL CS-Lactosylceramide BSA complex at 37°C, 5% CO₂, for 15 min, then chased at dye free medium for 10 min. NAADP treatment and confocal fluorescence recording were performed with a method described above. Fluorescence was recorded at excitation and emission wavelengths of 575 and 590 nm. The dynamic change of green fluorescence intensity in the regions of interest were analyzed by the Olympus Fluoview software (version 4.2, FV300).

BACKGROUND

- (1) Lysosomal NAADP-sensitive Ca²⁺ signaling is a newly-discovered intracellular pathway, which importantly participates in the regulation of cell functions in a wide variety of cell types.
- (2) Recent studies in our laboratory have demonstrated the identity of lysosomal NAADP-sensitive Ca²⁺ channel is TRP-ML1 in rat hepatocytes and bovine coronary myocytes (Zhang F and Li PL, *J Biol Chem*. 2007;282(35):25259-69, and Zhang F, Li PL, et al. *J Cell Mol Med*. 2008 Aug 27. [Epub ahead of print]).
- (3) It has been reported that TRP-ML1 channel proteins are predominantly expressed in the lysosomes and regulate membrane traffic and lipid metabolism, the mutation of TRP-ML1 can result in mucopolidiosis type IV, a neurodegenerative lysosomal storage disorder of which the clinical symptoms are illustrated by psychomotor retardation and ophthalmologic abnormalities.
- (4) The present study was designed to test whether NAADP acts on TRP-ML1 and contributes to a Ca²⁺-dependent lysosome and endosome fission process and thereby regulates lipid trafficking and metabolism in human fibroblasts.

RESULTS

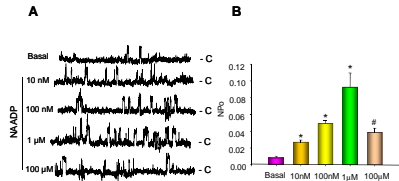


Fig. 1. The concentration-dependent effects of NAADP on the reconstituted lysosomal Ca²⁺ release channel activity by lipid bilayer assay. A: Representative recordings of single channel Cs⁺ currents before and after application of different doses of NAADP into the cis solution under the holding potential of +40 mV. B: Summarized data indicate that NAADP could increase lysosomal Ca²⁺ channel open probability from 0.00812 ± 0.00213 of basal level to 0.0565 ± 0.0117 at 100 nM NAADP, but decrease to 0.0213 ± 0.00526 after 100 μM NAADP was applied.

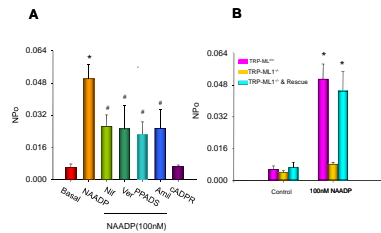


Fig. 2. Identity of lysosomal NAADP Ca²⁺ release channel is TRP-ML1. A: Pharmacologically, reconstituted lipid bilayer analysis shows that different L-type Ca²⁺ agonists of Nifedipine (Nif, 100 μM) and Verapamil (Ver, 100 μM), Na⁺ blocker of amiloride (Ami, 100 μM) or NAADP antagonist of PPADS (100 μM) could significantly decrease lysosome NAADP-sensitive Ca²⁺ channel open probability from 0.0511 ± 0.0073 of NAADP control group to 0.0265 ± 0.0057, 0.0253 ± 0.0114, 0.0256 ± 0.0092 and 0.0226 ± 0.0061 respectively. However, cADPR (100 nM) has no effects on the lysosomal Ca²⁺ channel activity. B: NAADP (100 nM) can activate lysosomal Ca²⁺ channel in lysosomal preparations from TRP-ML1^{+/+} fibroblast cells but not TRP-ML1^{-/-} fibroblasts. However, when the TRP-ML1^{-/-} fibroblasts rescued by TRP-ML1 transfection, the lysosomal NAADP-sensitive Ca²⁺ channel reactivity was recovered to that of TRP-ML1^{+/+} level. These results suggest that NAADP target lysosomal TRP-ML1 to release Ca²⁺.

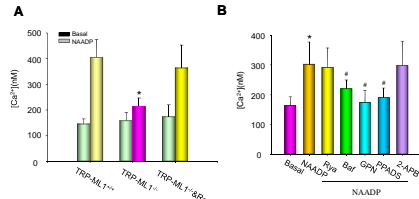


Fig. 3. Fluorescence Ca²⁺ image analysis of Ca²⁺ release by NAADP. A: NAADP (100 nM) could significantly release Ca²⁺ from TRP-ML1^{+/+} fibroblast cells from basal level of (nM) 180 ± 44.13 to 302.4 ± 74.28, however, this NAADP-induced Ca²⁺ release was almost abolished in TRP-ML1^{-/-} fibroblasts. While in the TRP-ML1 rescued TRP-ML1^{-/-} deficient cells, this NAADP-induced Ca²⁺ response was restored. B: Lysosomal function inhibitors of Bafilomycin A1 (Baf), GPN and NAADP antagonist could significantly attenuate NAADP-induced Ca²⁺ release in TRP-ML1^{+/+} fibroblasts. However, FR-Ca²⁺ channel antagonists of Rya (50 μM) and IP3/Ca²⁺ channel blocker of 2-APB has no inhibitive effects on NAADP-related Ca²⁺ responses. This functional results strongly suggests that TRP-ML1 is a critical component in the Ca²⁺ signaling for lysosomes in human fibroblasts.

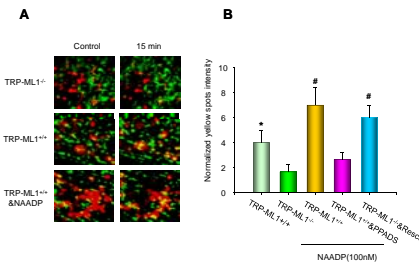


Fig. 4. NAADP-TRP-ML1 dependent interaction of endosomes and lysosomes in human fibroblast cells by confocal microscopic assay. A: Dextran-conjugated Rhodamine red shows the trace of lysosomes, Oregon green 488 indicates the endosomes and the yellow spots demonstrate the mixed content between endosomes and lysosomes. A: In TRP-ML1^{+/+} panel, the relative positions of endosomes and lysosomes have no obvious change during the 15 min chase, consistently, there are few yellow spots in 15 min image. However, in TRP-ML1^{-/-} panel, the interaction of endosomes and lysosomes are significantly increased, and the yellow spots intensity was subsequently enhanced. When TRP-ML1^{-/-} treated with NAADP, the yellow spots intensity in 15 min image were further elevated. B: The summary of normalized yellow spots density among different groups in 15 min image, which indicates that the presence of TRP-ML1 in fibroblasts could increase the endosomes and lysosomes interaction resulted in a significant increase of yellow intensity compared with that of TRP-ML1 deficiency cells. Consistently, when TRP-ML1 was rescued, the interaction of the two organelles restored. TRP-ML1 agonist of NAADP could further enhance yellow spot intensity in TRP-ML1^{-/-} cells, which could be attenuated by NAADP antagonist of PPADS. These results suggest that the dynamic interaction of endosomes and lysosomes is a NAADP-TRP-ML1 dependent process.

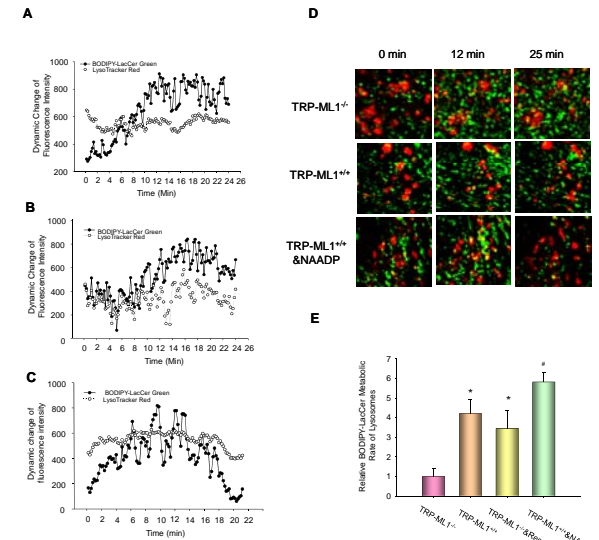


Fig. 5. NAADP increases BODIPY-LacCer green metabolic rate in lysosomes of human fibroblast cells. A, B, C: The BODIPY-LacCer green fluorescence intensity were dynamically increased from 0 to 12 min in all three groups of TRP-ML1^{+/+}, TRP-ML1^{-/-} and TRP-ML1^{-/-} + NAADP. However, in TRP-ML1^{-/-} fibroblasts, this increased green fluorescence intensity accumulate as a plateau, which suggest the metabolism of BODIPY-LacCer was blocked. In TRP-ML1^{-/-} fibroblasts, there was a decrease phase after the green fluorescence intensity reached apex, which indicates the metabolism of BODIPY-LacCer. This declined green fluorescence intensity phase was accelerated in TRP-ML1^{+/+} with NAADP treatment. D: Representative time sequential images at 0, 12 and 22 min. E: After normalizing to the Lyso-Tracker red intensity, the relative BODIPY-LacCer metabolic metabolism rates were quantified by dividing the change of green fluorescence intensity with time of occurrence. Summarized results show that metabolism rate of BODIPY-LacCer from 1 in TRP-ML1^{+/+} group to and in TRP-ML1^{-/-} group and TRP-ML1^{-/-} with treatment of 100 nM NAADP.

CONCLUSION

1. NAADP can function as an agonist of Lysosomal TRP-ML1 channel and release Ca²⁺ from lysosomes in human fibroblast cells.
2. Functionally, NAADP-activated lysosomal TRP-ML1 Ca²⁺ channel could promote a fusion-fission process of endosomes and lysosomes, and facilitate intracellular lipid trafficking and metabolism in lysosomes.