Reconstitution and Characterization of a Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP)-sensitive Ca$^{2+}$ Release Channel from Liver Lysosomes of Rats*

Fan Zhang and Pin-Lan Li

From the Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Nicotinic acid adenine dinucleotide phosphate (NAADP) is capable of inducing global Ca$^{2+}$ increases via a lysosome-associated mechanism, but the mechanism mediating NAADP-induced intracellular Ca$^{2+}$ release remains unclear. The present study reconstituted and characterized a lysosomal NAADP-sensitive Ca$^{2+}$ release channel using purified lysosomes from rat liver. Furthermore, the identity of lysosomal NAADP-sensitive Ca$^{2+}$ release channels was also investigated. It was found that NAADP activates lysosomal Ca$^{2+}$ release channels at concentrations of 1 nM to 1 μM, but this activating effect of NAADP was significantly reduced when the concentrations used increased to 10 or 100 μM. Either activators or blockers of Ca$^{2+}$ release channels on the sarcoplasmic reticulum (SR) had no effect on the activity of these NAADP-activated Ca$^{2+}$ release channels. Interestingly, the activity of this lysosomal NAADP-sensitive Ca$^{2+}$ release channel increased when the pH in cis solution decreased, but it could not be inhibited by a lysosomal H$^+$-ATPase antagonist, bafilomycin A1. However, the activity of this channel was significantly inhibited by plasma membrane L-type Ca$^{2+}$ channel blockers such as verapamil, diiltiazem, and nifedipine, or the nonselective Ca$^{2+}$, Na$^+$ channel blocker, amiloride. In addition, blockade of TRP-ML1 (transient receptor potential-mucolipin 1) protein by anti-TRP-ML1 antibody markedly attenuated NAADP-induced activation of these lysosomal Ca$^{2+}$ channels. These results for the first time provide direct evidence that a novel Ca$^{2+}$ release channel is present in the lysosome of native liver cells and that this channel is associated with TRP-ML1, which is different from ER/SR Ca$^{2+}$ release channels.

Nicotinic acid adenine dinucleotide phosphate (NAADP)$^2$ was first identified to mobilize Ca$^{2+}$ in sea urchin eggs (1, 2).

Now this signaling nucleotide has been shown to act as an endogenous regulator of intracellular Ca$^{2+}$ in a wide variety of cell types from plants to animals, participating in the regulation of cell functions (3–6). In some fractionation studies, NAADP-sensitive Ca$^{2+}$ stores were shown separable by density centrifugation from the endoplasmic reticulum (ER), one of the important intracellular Ca$^{2+}$-storing organelles that is sensitive to cyclic ADP-ribose (cADPR) and d-myo-inositol 1,4,5-trisphosphate (Ins(1,4,5)IP$_3$, IP$_3$) for Ca$^{2+}$ release. In other studies, it was found that the NAADP-sensitive stores are resistant to thapsigargin, an inhibitor of Ca$^{2+}$-ATPase in the ER or sarcoplasmic reticulum (SR). These results indicate that NAADP-induced Ca$^{2+}$ response may not be from the ER/SR stores (7, 8).

Recently, it has been demonstrated that NAADP-induced Ca$^{2+}$ release is associated with lysosomal function, because this Ca$^{2+}$ release response is dependent on a proton gradient maintained by an ATP-dependent vacuolar-type proton pump that is primarily present in lysosomes. Accumulating evidence shows that this NAADP-sensitive Ca$^{2+}$ store is unique and distinct from that of IP$_3$- and cADPR-sensitive Ca$^{2+}$ stores (9, 10). More recently, we have reported that NAADP has no direct effect on those reconstituted RyR/Ca$^{2+}$ release channels from the SR of coronary arterial smooth muscle and that a lysosome-associated Ca$^{2+}$ regulatory mechanism via NAADP contributes to ET-1-induced Ca$^{2+}$ mobilization in these arterial cells (11). In addition, ET-1-induced vasoconstriction of coronary arteries was dependent on an intact NAADP-lysosome signaling pathway. It is concluded that a novel Ca$^{2+}$ store associated with the lysosome that can be mobilized by NAADP is significantly implicated in the vasomotor response to ET-1 and thereby in the regulation of vascular tone. This lysosome-associated Ca$^{2+}$ store in arterial myocytes is functioning as a Ca$^{2+}$-triggering mechanism as confirmed in lysosomes of the sea urchin egg and pancreatic acinar cells (9, 12).

Functionally, lysosomes have originally been found to act as cell defenders to destroy foreign invaders, such as bacteria, and digest waste materials within the cells. It is generally accepted that TRP-ML1 (transient receptor potential-mucolipin 1)-mediated control of lysosomal Ca$^{2+}$ levels plays an important role in proper lysosome functions. TRP-ML1 is a nonselective cation channel that is permeable to Ca$^{2+}$ as well as to Na$^+$ and K$^+$. MOPS, 4-morpholinepropanesulfonic acid; Homo, homogenate; PM, plasma membrane.
Lysosomal NAADP-sensitive Ca\(^{2+}\) Release Channel, TRP-ML1

which is widely expressed and resides in the late endosomes/lysomes. The mutations of TRP-ML1 channel can cause mucolipidosis type IV disease, which is characterized by accumulation of lipids and membranous material in lysosomes, leading to developmental abnormalities of the brain and impaired neurological, ophthalmological, and gastric function (13–15). Lysosomal function currently has been extended as a store targeted by NAADP for Ca\(^{2+}\) release in a growing body of different cells. Similar to the nonselective cation channel properties of TRP-ML1, pharmacological evidence demonstrated that NAADP-sensitive Ca\(^{2+}\) release channel could also be blocked by L-type calcium channel inhibitors (diltiazem and dihydropyridines) and potassium channel blockers, including tetrahexylammonium (16). Although some recent studies reported different characteristics of TRP-ML1 channels in different cell lines or gene-transfected oocytes (13, 17–19), to our knowledge there has been no study that has characterized this TRP-ML1 channel in the lysosomes of native cells.

Lysosomes are reported to be abundant in the kidney and liver (20). It has been shown that extracts from rat liver can catalyze NAADP synthesis by exchanging nicotinamide of NADPH for nicotinic acid, and these extracts also have the ability to inactivate NAADP (21, 22). The cellular NAADP level has been detected in intact rat hepatocytes to be 109 ± 12 fmol/mg with the \(^{[32P]}\)NAADP binding assay. The NAADP-evoked Ca\(^{2+}\) release response has also been shown in microsomes from hepatocytes. The NAADP-elicted Ca\(^{2+}\) release, however, cannot be blocked by the inhibitors of either IP\(_{3}\) or ryanodine receptors (23). Furthermore, the cross-desensitization of IP\(_{3}\) and cADPR did not occur in liver microsomes, which suggested that the NAADP-mediated Ca\(^{2+}\) release was not through ER/SR Ca\(^{2+}\) stores (22).

In the present study, we characterized a lysosomal NAADP-sensitive Ca\(^{2+}\) release channel in liver cells and tested whether this channel was identical to TRP-ML1. First, we isolated and purified lysosomes from rat liver by percoll gradient centrifugation and reconstituted the lysosomal NAADP-sensitive Ca\(^{2+}\) release channels into a planar lipid bilayer. Then, we determined the biophysical and pharmacological characteristics of these channels. Our results demonstrated that the lysosomal NAADP-sensitive Ca\(^{2+}\) release channels are present in rat liver hepatocytes, and that these lysosomal NAADP-sensitive Ca\(^{2+}\) release channels are mediated by lysosomal TRP-ML1, which are different from RyR and IP\(_{3}\)R Ca\(^{2+}\) release channels on the ER/SR.

**EXPERIMENTAL PROCEDURES**

Isolation and Purification of Lysosomes—The rat liver lysosomes were isolated using the methods published previously (24–26) with a minor modification. Briefly, male Sprague-Dawley rats weighing 250–280 g, were fasted for 18 h and decapitated, and the livers were rapidly removed and cleaned with homogenization buffer (pH 7.4), which contained (in mM) 250 sucrose, 20 Tris-HCl, 1 phenylmethylsulfonfonyl fluoride (PMSF), 1 Na\(_{2}\)VO\(_{4}\), 1 mg/ml leupeptin, and 40 ml of HEPES with 1 tablet of complete proteinase inhibitor mixture (Roche Diagnostics GmbH, Germany). The livers were excised, minced well with scissors, suspended in 4 volumes of homogenates buffer, and homogenized first with 20 strokes by hand in a Dounce homogenizer, followed by 2 strokes in a Potter-Elvehjem tissue grinder rotating at 800 rpm. The homogenates, termed liver homo, were centrifuged at 600 \(\times\) g, 4 °C for 10 min, and the postnuclear supernatant was added 0.01 volume of 100 mM CaCl\(_{2}\), stock solution to a final [Ca\(^{2+}\)] of 1 mM, then incubated at 37 °C, for 5 min. Every 2.7 ml of incubated mixture was layered on 22.3 ml of iso-osmotic (0.25 m sucrose) Percoll at a density of 1.08 g/ml in a Beckman type 45Ti rotor tube, and centrifuged at 60,000 \(\times\) g, 4 °C for 15 min. After centrifugation, 4 fractions of solution from the bottom of the tube was carefully collected and pooled, termed homo percoll, then centrifuged in Beckman 32Ti type SW rotor tube at 100,000 \(\times\) g, 4 °C for 1 h. A broad turbid layer (termed: lyso layer) in the bottom of the tube was carefully collected and diluted with 2 volumes of homogenization buffer; the remaining supernatant solution above the broad turbid layer (termed: upper layer) was discarded. Then, the diluted lysosome suspension was centrifuged at 10,000 \(\times\) g for 30 min, and the pellet was washed twice by centrifugation under the same conditions. The washed pellet was finally suspended in the resuspending solution (0.9% NaCl, 0.3 m sucrose) with 100 μM PMSF, and used as the purified lysosomal fraction.

Biochemical Identification of Lysosomes—Acid phosphatase is one of the acid hydrolases that normally resides in lysosomes. It is a classical marker for the identification of lysosomes in subcellular fractions. The purity of the prepared lysosomes from liver was determined by the acid phosphatase assay kit from Sigma, and the acid phosphatase activity was represented by the conversion of 4-nitrophenyl phosphate to 4-nitrophenol and measured by spectrophotometer at 405 nm. Liver plasma membrane, endoplasmic reticulum, and liver homogenates were used as controls.

The preparation of liver plasma membrane and endoplasmic reticulum by a method described previously with a minor modification (27, 28). Briefly, the liver homogenate from lysosome fraction isolation process was centrifuged at 1000 \(\times\) g, 4 °C for 10 min. The nuclear pellet was discarded, and the postnuclear supernatant was collected and centrifuged at 10,000 \(\times\) g, 4 °C for 25 min to obtain the postmitochondrial supernatant. The postmitochondrial supernatant was further centrifuged at 78,000 \(\times\) g, 4 °C for 15 min. The microsomal pellet was collected and subfractionated by discontinuous sucrose density centrifugation. The microsomal pellet was first resuspended in sucrose solution to a final mixture contained 53% sucrose and 1–2 mg/ml microsomal protein; then 10 ml of this mixture was overlaid with 10 ml of 34% sucrose, then 10 ml of 30% sucrose, followed by 8 ml of 0.25 m sucrose and centrifuged at 78,000 \(\times\) g for 16 h at 4 °C in a swinging bucket rotor (Beckman type SW–27). All sucrose solutions were buffered with Tris–HCl (5 \(\times\) \(10^{-3}\) M, pH 8.0). After centrifugation, the endoplasmic reticulum fraction at the top of 53% sucrose layer and the plasma fraction at the top of 34% sucrose layer were collected respectively, washed with Tris–HCl buffer (5 \(\times\) \(10^{-3}\) M, pH 8.0) and harvested by centrifugation at 78,000 \(\times\) g, 4 °C for 90 min. Each of the fractions was then suspended in a convenient volume of Tris–HCl buffer for use.
Lysosomal NAADP-sensitive Ca\(^{2+}\) Release Channel, TRP-ML1

The purity of endoplasmic reticulum preparations was analyzed by the cytochrome c reductase (NADPH) assay kit from Sigma. Eukaryotic NAADP-cytochrome c reductase is a flavoprotein localized to the endoplasmic reticulum, which is widely used as an endoplasmic reticulum marker. Cytochrome c reductase (NADPH) activity was represented by the reduction of cytochrome c in the presence of NADPH and measured by spectrophotometer with a kinetic program at 550 nm, 25 °C. Plasma membrane identity was determined by the alkaline phosphodiesterase activity, which was used as a marker enzyme for the plasma membrane with a method described previously (27, 29–31).

Western Blot Analysis—Identification of lysosomes was further determined by Western blot analysis using an antibody of LAMP2 (lysosome-associated membrane protein 2), a lysosomal specific marker, with the method described previously (32). The existence of TRP-ML1 in lysosome was also detected with corresponding antibody. Furthermore, the plasma membrane protein, caveolin, was examined to rule out the contamination possibility of plasma membrane in purified lysosomes. During probing, the concentrations of antibodies were used according to the manufacturer’s instructions. LAMP2 and TRP-ML1 antibodies were purchased from Abcam\(^{®}\), and Caveolin antibody from BD Transduction Laboratories, respectively. The corresponding bands for LAMP2, TRP-ML1, and caveolin were visualized at 110, 65, and 24 kDa, respectively.

Reconstitution of Lysosomal Channels into the Lipid Bilayer and Biophysical Characterization of the NAADP-activated Ca\(^{2+}\) Release Channels—The purified lysosomal membranes from rat liver were reconstituted into planar lipid bilayers with the method described previously (11, 33, 34). Briefly, phosphatidylethanolamine and phosphatidyserine (1:1) (Avanti Polar Lipids, Alabaster, AL) were dissolved in decane (25 mg/ml) and used to form a planar lipid bilayer in a 250-μm aperture between two chambers filled with cis and trans solutions. After the lipid bilayer was formed, lysosomal membrane preparations (40–60 μg) were added to the cis side solution. The force of driving the lysosomal NAADP channel-containing membranes into the lipid bilayer and promoting their fusion was the electrochemical gradients of Cs\(^+\) between the cis and trans side. The success rate for reconstitution of lysosomal Ca\(^{2+}\) release channel was about 60% of paintings. However, the success rate was dependent on the quality of the lysosomal preparations and the concentrations of frozen stored aliquot lysosomal preparations. High quality and high concentrations of lysosome preparations could ensure higher success rates in reconstitution of the lysosomal channels into the lipid bilayer. In these reconstitution experiments, Cs\(^+\) was used as the charge carrier. Axopatch 200B amplifier (Axon instruments) was applied for the bilayer potential control and currents recording. Data acquisition and analysis were performed with pCLAMP software (version 9, Axon Instruments). The channel open probability (NP\(_{0}\)) in the lipid bilayer was determined from 3 to 5 min recordings, and all lipid bilayer experiments were performed at room temperature (~20 °C). The Ca\(^{2+}\) release channel activity was detected in a symmetrical 300 mM cesium methanesulfonate and 10 mM MOPS solution (pH 7.2). To establish current-voltage relationship of lysosomal NAADP-activated Ca\(^{2+}\) release channels, the single channel currents were recorded, while holding potentials were varied from −40 to +40 mV in steps of 20 mV.

Pharmacological Characterization of NAADP-sensitive Ca\(^{2+}\) Release Channels—First, we investigated the dose effects of NAADP on the reconstituted lysosomal Ca\(^{2+}\) release channel activity; Second, we examined the effects of various plasmalemmal Ca\(^{2+}\) channel activators on the reconstituted lysosomal channels and then addressed whether NAADP-induced activation can be blocked by any Ca\(^{2+}\) channel inhibitors or blockers. A low concentration of Rya (2 μM) and 1 μM IP\(_{3}\) were used as activators of RyR and IP\(_{3}\)R, and a concentration of Rya (50 μM) and 2-aminophenoxydiphenylborate (2-APB, 100 μM) as blockers of these SR/ER Ca\(^{2+}\) channels. Three different blockers of cell membrane voltage-dependent Ca\(^{2+}\) channel, diltiazem (100 μM), verapamil (100 μM), nifedipine (100 μM), and a non-selective Ca\(^{2+}\) channel blocker, amiloride (1 mM) were used. Bafilomycin A1 (Baf, 100 nM), an ATP-dependent vacuolartype proton pump inhibitor was used to test whether the activity of these reconstituted lysosomal channels is associated with the ATP proton pump on lysosomes, In addition, the effects of pH on the NAADP-sensitive Ca\(^{2+}\) release channel activity was examined in the presence of 10 nM NAADP. All of these compounds were added into the cis solution, and currents were recorded at holding potentials of +40 mV.

Planar Lipid Bilayer Analysis of NAADP Effect on ER RyR/ Ca\(^{2+}\) Release Channels—The preparation of ER-enriched microsomes from rat liver and reconstitution of these ER membranes into a planar lipid bilayer were performed by the same protocol described in the lysosomal channel reconstitution section, with cesium used as the charge carrier. The Ca\(^{2+}\) release channel activity was detected in a symmetrical 300 mM cesium methanesulfonate and 10 mM MOPS solution (pH 7.2). The effects of NAADP (10 nM) on the activity of RyR Ca\(^{2+}\) (RyR/ Ca\(^{2+}\)) release channels of the ER were determined, and the positive control with cADPR (10 nM) and its antagonist of Rya (50 μM) were used to validate the experimental condition. All doses of these compounds were based on previous studies (11, 33), and all compounds were added into the cis solution, with currents recorded at a holding potential of +40 mV.

Identity of NAADP-sensitive Ca\(^{2+}\) Release Channels—Because both NAADP-sensitive Ca\(^{2+}\) release channel and TRP-ML1 present in lysosomes, previous studies demonstrated that they shared similar pharmacological properties (14, 16). By using the reconstituted lipid bilayer methods, we further addressed whether the two channels were identical. We used an anti-TRP-ML1 polyclonal antibody (sc-26269, Santa Cruz Biotechnology), which was raised in goat against a peptide map of the C terminus of TRP-ML1 of mouse origin, to block the TRP-ML1 ion channel from lysosomes in the reconstituted bilayer. In these experiments, a serial diluted TRP-ML1 antibody was added to the bath solution at final concentrations of 1:5000, 1:500, and 1:50 for 5 min respectively, and then 500 nM NAADP was added. Before and after addition of NAADP, the channel currents were recorded at a holding potential of +40 mV. Normal goat serum was used as the substitute for goat polyclonal TRP-ML1 antibody for control experiments.
Lysosomal NAADP-sensitive \( Ca^{2+} \) Release Channel, TRP-ML1

FIGURE 1. The purity of lysosomes confirmed by measurements of the marker enzyme activity. Panel A shows that the conversion rate of 4-nitrophenyl phosphate to 4-nitrophenyl by lysosome marker enzyme of acid phosphatase in liver homogenates (Homo), lysosome (Lyso), endoplasmic reticulum (ER), and plasma membrane (PM), respectively. Panel B shows the reduction of cytochrome c by SR/ER marker enzyme NADPH-cytochrome c reductase in the presence of NADPH. Panel C shows the conversion of sodium thymidine 5’-monophosphate p-nitrophenyl ester to p-nitrophenyl by plasma membrane marker enzyme alkaline phosphodiesterase. Panel D, Western blot analysis demonstrating that the purified Lyso were recognized by the antibody against either LAMP2 or TRP-ML1, but not by caveolin antibody. *, significant difference from other fractions (\( p < 0.05, n = 6 \)).

Statistics—Data are presented as means ± S.E.; the significance of the differences in mean values between and within multiple groups was examined using an analysis of variance for repeated measures followed by a Duncan’s multiple range test. A Student’s \( t \) test was used to evaluate statistical significance of differences between two paired observations. \( p < 0.05 \) was considered statistically significant.

RESULTS

Purification and Identification of Lysosomes—The purity of lysosomes was demonstrated by determining some marker enzyme activity. As shown in Fig. 1A, the conversion rate of 4-nitrophenyl phosphate to 4-nitrophenyl by lysosome marker enzyme, acid phosphatase, were 0.98 ± 0.25, 15.33 ± 1.23, 0.59 ± 0.33, and 0.40 ± 0.27 \( \mu \)mol/h/mg protein in liver homogenates (Homo), lysosome (Lyso), endoplasmic reticulum (ER), and cell plasma membrane (PM) preparations, respectively. Fig. 1B shows that the reduction of cytochrome c by an ER marker enzyme, NADPH-cytochrome c reductase in the presence of NADPH was almost undetectable in the lysosome preparation. Similarly, Fig. 1C shows that the conversion of sodium thymidine 5’-monophosphate p-nitrophenyl ester to p-nitrophenyl by a cell plasma membrane marker enzyme, alkaline phosphodiesterase. The alkaline phosphodiesterase activity in lysosome preparation was 26.1 and 3.31 times lower than that in PM and Homo preparations, respectively. These results indicate that the isolated lysosomes are highly purified and free of ER and cell membrane contamination.

In Fig. 1D, Western blot analysis shows that the purified lysosome (Lyso) fraction was recognized by antibodies against LAMP2, a lysosome-specific protein, and TRP-ML1, a known non-selective \( Ca^{2+} \) release channel, but not by caveolin antibody. In contrast, in cell membrane preparations, neither LAMP2 nor TRP-ML1 were detectable. However, in the ER preparation, there was no protein that could be recognized by any of these three antibodies.

Characterization of the Reconstituted Lysosomal \( Ca^{2+} \) Release Channels in the Planar Lipid Bilayer—Fig. 2A shows representative recordings of \( Ca^{2+} \) channel currents at holding potentials from -40 to +40 mV with symmetrical 300 mM cesium in cis and trans solution. Fig. 2B summarizes the relationship of holding potential and the channel current amplitude. It is clear that the channel current increased with the enhancement of holding potential. By calculation, the mean conductance for these lysosomal \( Cs^{+} \) currents was 174 pS with a reversal potential of ~0 mV. When \( Cs^{+} \) gradients between cis/trans solution were changed from 300/300 mM to 200/300 mM or 300/200 mM, the equilibrium potential correspondingly varied from 0 mV to -9.2 or +9.5 mV, which was almost equivalent to the theoretical value of ±10.2 mV based on the Nernst equation. In addition, the \( NP_{0} \) of these \( Cs^{+} \) currents increased when the bilayer holding potential increased from 0 to +40 mV or decreased from 0 to -40 mV (data not shown). However, this voltage dependence is dependent on the magnitude of holding potential rather than on the polarity of the clamp voltage.

Next, we examined whether these channels can be activated by NAADP because the major goal of the present study was to explore the mechanism by which this novel signaling nucleotide causes intracellular \( Ca^{2+} \) release through lysosomes. Fig. 3A depicts the representative recordings of single-channel \( Cs^{+} \) currents before and after addition of NAADP into the cis/trans solutions, and Fig. 3B summarizes the results showing that NAADP from 1 nM to 1 \( \mu \)M markedly increased the openings of these reconstituted lysosomal \( Ca^{2+} \) channels. The \( NP_{0} \) of these channels increased from 0.0112 ± 0.0013 of control to 0.2022 ± 0.0249 and 0.2225 ± 0.0438, respectively at 0.5 and 1 \( \mu \)M NAADP, but when NAADP concentrations were further
increased to 10 μM and 100 μM, the channel NP0 was decreased to 0.0907 ± 0.023 and 0.0323 ± 0.0267, respectively. Meanwhile, when the lysosomal preparations were preincubated with 0.5 nM subthreshold NAADP for 5 min, then 1 μM NAADP-induced increase in channel activity was substantially attenuated. In addition, when NAADP was added into the trans-side solution, the channel activation effects were not observed (data not shown). These results indicate that the reconstituted lysosomal Ca2+ release channels are sensitive to NAADP, and therefore it may be a target for the action of NAADP to mobilize intracellular Ca2+.

Effects of IP3, Rya, and 2-APB on Lysosomal Ca2+ Release Channel Activity—To determine whether the reconstituted lysosomal Ca2+ release channels are characteristic of two other well known intracellular Ca2+ release channels, namely IP3- and ryanodine-sensitive SR/ER channels, several groups of experiments with pharmacological interventions were performed in lipid bilayer reconstitution preparations. First, the agonists of RyR and IP3R were used to test whether they can activate these reconstituted lysosomal Ca2+ release channels. The representative recordings depicting the effects of IP3 and Rya on the activity of reconstituted lysosomal NAADP-sensitive Ca2+ release channels are presented in Fig. 4A, and summarized results in Fig. 4B show that the NP0 of these channels was 0.0134 ± 0.0048 in the presence of a low concentration of Rya (2 μM), which was not significantly different from 0.0131 ± 0.0047 of control. Rya at this concentration has been reported to significantly activate the RyR/Ca2+ release channels on the SR/ER preparations (11, 33, 34). Moreover, when reconstituted channels were stimulated by 1 μM of IP3, another Ca2+ release agonist of SR/ER, the NP0 of these channels was 0.0129 ± 0.0044, which was also similar to the control. In additional group of experiments, we tested the effects of different SR/ER Ca2+ release channel blockers on the activity of reconstituted NAADP-sensitive lysosomal Ca2+ release channels. A high dose of Rya (50 μM) as the inhibitor of RyR/Ca2+ release channels or 2-APB (100 μM) as the antagonist of IP3R had no significant inhibitory effects on the NP0 of reconstituted lysosomal Ca2+ channels, which was stimulated by NAADP (Fig. 5). In addition, bafilomycin A1, an inhibitor of H+−ATPase that is
commonly used to inhibit lysosomal function, did not change the activity of these channels in the presence of NAADP (Fig. 5). All these inhibitors alone have no effect on the channel open probability compared with control (data not shown). These results confirm that NAADP-activated lysosomal Ca\(^{2+}\) release channels are different from those channels present on the SR/ER.

**Effects of Classical Ca\(^{2+}\) Channel Blockers on NAADP-sensitive Lysosomal Ca\(^{2+}\) Channel Activity**—Fig. 6A presents representative current recordings after the reconstituted bilayer membrane was treated by NAADP (10 nM), or nifedipine (Nif, 100 \(\mu M\)), diltiazem (Dil, 100 \(\mu M\)), verapamil (Ver, 100 \(\mu M\)), or amiloride (Amilo, 1 mM) plus NAADP (10 nM). In Fig. 6B, summarized results show that NAADP-induced activation of reconstituted lysosomal Ca\(^{2+}\) release channels was markedly attenuated by three plasma membrane Ca\(^{2+}\) channel blockers or a nonselective Ca\(^{2+}\), Na\(^{+}\) channel blocker, amiloride.

**Effects of pH on Lysosomal Ca\(^{2+}\) Release Channel Activity**—Fig. 7A shows the representative recordings of lysosomal NAADP-sensitive Ca\(^{2+}\) release channels after sulfuric acid was added into the cis solution to adjust pH value from 7.2 to 6.0, 4.0 and 2.0 in the presence of 10 nM NAADP, respectively. Fig. 7B summarized results indicating that NAADP-induced increase in channel NP\(_0\) was significantly enhanced, from 0.0802 ± 0.0121 at pH 7.2 to 0.1069 ± 0.0121 at pH 6, 0.1317 ± 0.0198 at pH 4, and 0.1773 ± 0.0401 at pH 2.

**Effects of NAADP on the Activity of Reconstituted RyR/Ca\(^{2+}\) Release Channels from Rat Liver ER**—Fig. 8 shows the effects of NAADP, cADPR, or Rya plus cADPR on the activity of the reconstituted RyR/Ca\(^{2+}\) release channels in the planar lipid bilayer. Panel A, representative RyR/Ca\(^{2+}\) channel recording traces are presented, showing no changes in channel opening in the presence of NAADP. Panel B summarizes the results showing that NAADP had no effects on the NP\(_0\) of the channels. However, cADPR, a well-known SR RyR/Ca\(^{2+}\) release channels agonist, significantly increased the NP\(_0\) of the channels. When the bilayer was pretreated with RyR/Ca\(^{2+}\) channel antagonist of 50 \(\mu M\) Rya,
cADPR-induced channel activity was abolished. These results suggest that NAADP has no direct effect to activate RyR/Ca\(^{2+}\)/H\(_{11001}\) channels on the ER from rat liver, as does cADPR.

**Effects of TRP-ML1 Protein Blockade on Lysosomal NAADP-sensitive Ca\(^{2+}\)/H\(_{11001}\) Release Channel Activity**—To determine the identity of lysosomal NAADP-sensitive Ca\(^{2+}\)/H\(_{11001}\) channel, the channel activity was determined in TRP-ML1 protein-blocked lysosomal preparation with the planar lipid bilayer. Fig. 9A presents some representative current recordings of reconstituted lysosomal NAADP-sensitive Ca\(^{2+}\) release channel under control or in TRP-ML1 protein-blocked lysosome preparation. As summarized in Fig. 9B, TRP-ML1 polyclonal antibody could dose-dependently attenuate 500 nM NAADP-induced increase in lysosomal Ca\(^{2+}\) release channel activity. The NP\(_0\) of these reconstituted lysosomal Ca\(^{2+}\) release channels decreased from 0.1954 ± 0.0189 of the normal goat serum (NGS) group to 0.1579 ± 0.0191, 0.0542 ± 0.0133 and 0.0224 ± 0.0077 of groups of lysosomal membrane preparations pretreated with 1:5000, 1:500, and 1:50 anti-TRP-ML1 antibody, respectively. However, the nonspecific goat serum had no inhibitory effects on the NAADP-activated Ca\(^{2+}\) release channel activity. It was also found that addition of the TRP-ML1 antibody into trans solution had no effect on the NAADP-induced activation of lysosomal channels (data not shown).

**DISCUSSION**

The present study provides the first direct evidence that a Ca\(^{2+}\) release channel is present in the lysosomes of native liver tissue. This channel may be the target for the action of NAADP, a most potent intracellular Ca\(^{2+}\)-mobilizing nucleotide. This lysosomal NAADP-sensitive Ca\(^{2+}\) channel had distinct characteristics from ER/SR Ca\(^{2+}\) release channels. The identity of this NAADP-activated Ca\(^{2+}\) release channel had been demon-
Lysosomal NAADP-sensitive Ca$^{2+}$ Release Channel, TRP-ML1

Stratified to be TRP-ML1, which is responsible for the action of NAADP in mobilization of intracellular Ca$^{2+}$ through lysosomes (9, 10, 12).

In our experiments, a Cs$^+$ current was recorded by directly reconstituting lysosomal preparation into lipid bilayer. This current was confirmed in a number of similar studies from our laboratory and by others as a Ca$^{2+}$ channel (33–35). Although we also recorded channel activities with Ca$^{2+}$ as a charge carrier ion in these lysosomal preparations, the use of Cs$^+$ as a charge carrier ion largely stabilized the current for biophysical and pharmacological characterization. This substitution of Cs$^+$ for Ca$^{2+}$ as a charge carrier was widely used for reconstitution of channels and in studies on their pharmacological characteristics and physiological regulation of intracellular organelar channels (36–38).

One of the important findings in the present study was that NAADP could activate these reconstituted Ca$^{2+}$ release channels from purified lysosomes. The results from the present bilayer studies also demonstrated that high concentrations of NAADP had desensitizing effects on the lysosome Ca$^{2+}$ release channel activity. In addition, pretreatment of the bilayer with a subthreshold concentration of NAADP also substantially attenuated the effect of a subsequent high dose of NAADP. This suggests that there is self-desensitization mechanism that occurs in this NAADP-activated Ca$^{2+}$ release channel. This self-desensitization property of channels has now been widely used as a diagnostic tool to confirm the action of NAADP as Ca$^{2+}$ releasing second messenger (39–42). These results support the view that NAADP is able to mobilize Ca$^{2+}$ from a lysosome store into the cytoplasm.

However, in some previous studies in T-lymphocytes and other cells, NAADP has been demonstrated to activate the RyR Ca$^{2+}$ release channels on the SR/ER. In those experiments, RyR blockade attenuated NAADP-induced Ca$^{2+}$ release in whole cell preparations (6). Unfortunately, these studies did not show whether the action of NAADP on the RyR/Ca$^{2+}$ release channel activity is primary or secondary. In this regard, there is considerable evidence that NAADP-induced Ca$^{2+}$ release usually operates in combination with other pathways or factors, and it

FIGURE 8. Effects of NAADP on the activity of reconstituted RyR/Ca$^{2+}$ release channel of ER from rat liver in planar lipid bilayer. A, representative recordings of reconstituted RyR/Ca$^{2+}$ release channels under control conditions and after addition of NAADP (10 nM), cADPR-ribose (cADPR, 10 nM), or ryanodine (Rya, 50 μM) plus cADPR (10 nM) into the cis side solution at a holding potential of 40 mv. B, summarized data showing the NP0 of the reconstituted RyR/Ca$^{2+}$ channels under different treatments. *, significant difference from control, NAADP, and Rya plus cADPR group (p < 0.05, n = 6 bilayers from 6 rats).

FIGURE 9. Effects of anti-TRP-ML1 antibody on lysosomal NAADP-sensitive Ca$^{2+}$ release channel activity in the planar lipid bilayer. A, representative current recordings of reconstituted lysosomal NAADP-sensitive Ca$^{2+}$ release channel currents under basal level, normal goat serum (NGS) or after anti-TRP-ML1 antibody treatment. B, summarized data showing the inhibitive effects of anti-TRP-ML1 antibody on the NAADP-induced channel activity. *, significant difference from NGS-treated group. (p < 0.05, n = 8 bilayers from 6 rats).
is often observed that the resultant Ca\(^{2+}\) signals evoked by NAADP are boosted by Ca\(^{2+}\) release via activation of RyRs, IP\(_3\)Rs, or both (2, 8, 10, 43, 44). Evidence from our current channel reconstitution study indicated that agonists and antagonists of SR/ER RyR/Ca\(^{2+}\) release channels had no effects on lysosomal NAADP-sensitive Ca\(^{2+}\) release channel activity and that NAADP has no direct effect to activate RyR/Ca\(^{2+}\) channels on the ER. This suggests that NAADP activates lysosomal Ca\(^{2+}\) release channel and thereby releases small amount of Ca\(^{2+}\) into cytosol to trigger global Ca\(^{2+}\) increase in the cytoplasm via CICR.

Interestingly, we found that bafilomycin A1, a common inhibitor of lysosome function, had no effect on the activity of reconstituted NAADP-sensitive Ca\(^{2+}\) release channels. This is not consistent with the observations in intact cells such as pancreatic acinar cells (45), pulmonary arterial myocytes (46), neutrite (47), and coronary arterial myocytes (11), where bafilomycin A1 selectively inhibited NAADP-induced Ca\(^{2+}\) release in the lysosomes. The reason for this difference may be associated with the mechanisms mediating lysosome Ca\(^{2+}\) stores, which may be revealed differently in intact cells or isolated lysosomes of these cells. In intact cells, lysosomes remained to be an intact organelle and bafilomycin A1-inhibitable V-H\(^+\)-ATPase acts to drive Ca\(^{2+}\) uptake by Ca\(^{2+}\)/H\(^+\) exchange. Therefore, bafilomycin A1 inhibits V-H\(^+\)-ATPase and leads to the depletion of Ca\(^{2+}\) storage in their lysosomes. With Ca\(^{2+}\) depletion from lysosomes, NAADP-induced Ca\(^{2+}\) triggering release and late robust mobilization of Ca\(^{2+}\) from the SR/ER are subsequently blocked. However, in the reconstituted system with purified lysosomes used in the present study, only partial lysosome membrane rather than the whole lysosome was incorporated into the lipid bilayer, and therefore there is no Ca\(^{2+}\) store or depletion issue in this preparation because the concentration of Ca\(^{2+}\), a carrier charge ion of Ca\(^{2+}\), between both sides of bilayer membrane is constant. Although under this condition bafilomycin A1 may still have effect on V-H\(^+\) ATPase activity, there is no effect on Ca\(^{2+}\) store for channel activity.

The ability of pH decrease to enhance the NAADP-sensitive Ca\(^{2+}\) release channel activity demonstrated in our experiments is in contrast to the results obtained from Ca\(^{2+}\) release experiments using hepatocyte microsomes (22), where NAADP-induced Ca\(^{2+}\) releases were not affected by the pH changes in the incubation medium from 6.4 to 7.8. This discrepancy may be related to the use of different pH ranges. The pH range applied in our experimental protocols was from 7.2 to 2, which cover the physiological pH value (~4) in lysosomal compartment, where lysosome Ca\(^{2+}\) release channel was activated by NAADP. In addition, in our reconstituted bilayer experiments, the purified lysosomes were used, which may lead to different results from unpurified tissue homogenates or microsomes, in which in fact a mixture of Ca\(^{2+}\) stores exists.

The present findings that L-type Ca\(^{2+}\) channel blockers reduced NAADP-sensitive Ca\(^{2+}\) release channel activity suggest that this lysosomal channel is pharmacologically similar to the cell plasmalemmal Ca\(^{2+}\) channels to some extent. This is consistent with previous results obtained from intact cells that plasmalemmal Ca\(^{2+}\) channel blockers considerably depressed the NAADP-induced Ca\(^{2+}\) release response (16, 48, 49).

Although there was a concern over the possible contamination of lysosome preparations by plasma membrane, our results did not support this assumption because caveolin-1, a specific plasmalemmal marker, was not detectable in our purified lysosomes. Enzyme assay also confirmed cell membrane specific enzyme activity could not be detected in these lysosome preparations. Based on these results, it is impossible that these reconstituted channels are contaminated by cell plasma membrane although they share some pharmacological feature of L-type Ca\(^{2+}\) channels.

Next, we explored another possibility for the identity of this lysosomal NAADP-sensitive Ca\(^{2+}\) release channels to be TRP-ML1. TRP-ML1 is a member of TRP (transient receptor potential) family that comprises more than 30 non-selective cation channel blockers considerably depressed NAADP-induced lysosomal channel activity. This suggests that TRP-ML1 may mediate the activity of NAADP-sensitive Ca\(^{2+}\) release channels in lysosomes. To test this possibility, we used a TRP-ML1 specific polyclonal antibody to examine if the channel activity could be blocked. It has been reported that TRP-ML1 wide-type is a 580-amino acid protein with six transmembrane domains and the C terminus of this channel protein is the feature functional domain of the transient receptor potential cation channel family (19, 51). The anti-TRP-ML1 antibody used in the current experiments was raised against a peptide mapping at the C terminus of TRP-PL1 of mouse origin. It was found that anti-TRP-ML1 antibody could dose-dependently attenuate NAADP-induced lysosomal channel activity. This suggests that TRP-ML1 may be the identity of lysosomal NAADP-sensitive Ca\(^{2+}\) release channels. In regard to TRP-ML1 channel blockers, several studies have used amiloride for characterization of TRP-ML1 channel activity, although it has often been used as a sodium channel blocker, such as epithelial Na\(^{+}\) channel (ENaC) (18, 52, 53). Interestingly, this compound substantially blocked NAADP-induced activation of the channels reconstituted in the lipid bilayer. Taken together, it is believed that TRP-ML1 may mediate NAADP-induced activation of a lysosomal Ca\(^{2+}\) release channel and thereby participates in the regulation of intracellular Ca\(^{2+}\) levels.

Our result that TRP-ML1 may serve as a lysosomal NAADP-sensitive Ca\(^{2+}\) release channel is different from a report that NAADP was not likely to regulate mucolipin-1 (TRP-ML1) channel, which was based on overexpressed mucolipin-1 showing no increase in the radiolabeled NAADP binding in NRK cells (54). It is obvious that the results from native TRP-ML1 in our experimental preparation might not be comparable to those from exogenously overexpressed genes. In addition, it should be noted that the action of NAADP through TRP-ML1 channels indicated by our results unnecessarily means that this protein is the receptor for this second messenger. In this context, there was some evidence that mucolipin-1 is able to oligomerize and/or form complexes with other proteins (55, 56), and this heteromeric formation to constitute cation-permeable pores is common in TRP channels (57). Obviously, how NAADP acts to activate TRP-ML1 channels in our preparations remains to be clarified. The possible mechanisms may be related to direct binding to TRP-ML1 or stimulation of some...
Lysoosomal NAADP-sensitive Ca\(^{2+}\) Release Channel, TRP-ML1

regulatory proteins, as we and others confirmed for the actions of cADPR on RyR/Ca\(^{2+}\) channels on the SR (34, 58, 59).

Our findings that TRP-ML1 may act as a NAADP-sensitive Ca\(^{2+}\) release channel and mediate Ca\(^{2+}\) release from lysosomes is consistent with previous studies that TRP-ML1 is a non-selective Ca\(^{2+}\)-permeable channel that may regulate lysosomal Ca\(^{2+}\) release and consequently agonist-evoked Ca\(^{2+}\) signals (15, 60). However, recent studies have indicated that TRP-ML1 also functions as monovalent-permeable and H\(^{+}\)-selective ion channel that leads to H\(^{+}\) leak in lysosomes and thereby prevents overacidification in these organelles (13, 18). There are several possibilities for this discrepancy. First, it is possible that this variance regarding the TRP-ML1 channel properties may be due to different channel recording methods. In studies indicating that TRP-ML1 is a H\(^{+}\)-permeable channel, was based on measuring whole cell currents in HEK293 cells expressing TRP-ML1. Although whole cell recording configuration could be used to characterize a channel, those studies did not provide a more accurate channel characterization configuration, namely single channel recording mode to record channel activity. In the present study, we did use single channel recording mode to identify the properties of these channels, which depends on the existence of TRP-ML1. Second, previous studies overexpressed TRP-ML1 in HEK293, which mainly were presented in the plasma membrane. The recoded channel currents were not directly derived from endogenous lyosomal TRP-ML1 channels as recorded in our studies. The location of TRP-ML1 may produce different ion permeabilities. Third, the channel recordings in the present study were performed using lysosomes from native liver tissue or cells, which are different from other previous studies that primarily used cell lines for TRP-ML1 channel characterization (13, 14, 18, 60), including the studies that indicated H\(^{+}\) permeability of TRP-ML1. It is also possible that TRP-ML1 expression may produce different phenotypes in cell lines and native cells. Finally, there may be possible species differences in the expression of TRP-ML1 channel characteristics. Almost all previous studies were done in human cell lines or in oocytes with expressed genes (13, 18). It is not surprising that the TRP-ML1 channels expressed in different cell types from different species may give different ion permeabilities, given the nonselectivity of these channels to ions.

In summary, the present study, for the first time, characterized an NAADP-sensitive lysosomal Ca\(^{2+}\) release channel from native liver tissue or cells through lipid bilayer reconstitution. This channel is different from IP\(_3\)R or RyR on SR/ER, but shares some features of the plasma membrane L-type Ca\(^{2+}\) channels. A nonselective cation permeable channel, TRP-ML1, is confirmed to be the identity of this NAADP-sensitive lysosomal Ca\(^{2+}\) release channel.

REFERENCES
13. Kiseloy, K., Chen, J., Rabbi, Y., Oberdick, D., Tjon-Kon-Sang, S., Shche-

Downloaded from www.jbc.org at VIVA, VA Commonwealth Univ on December 13, 2007