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Release of calcium from endolysosomes increases calcium influx through N-type calcium channels: Evidence for acidic store-operated calcium entry in neurons

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Abstract

Neurons possess an elaborate system of endolysosomes. Recently, endolysosomes were found to have readily releasable stores of intracellular calcium; however, relatively little is known about how such ‘acidic calcium stores’ affect calcium signaling in neurons. Here we demonstrated in primary cultured neurons that calcium released from acidic calcium stores triggered calcium influx across the plasma membrane, a phenomenon we have termed “acidic store-operated calcium entry (aSOCE)”. aSOCE was functionally distinct from store-operated calcium release and calcium entry involving endoplasmic reticulum. aSOCE appeared to be governed by N-type calcium channels (NTCCs) because aSOCE was attenuated significantly by selectively blocking NTCCs or by siRNA knockdown of NTCCs. Furthermore, we demonstrated that NTCCs co-immunoprecipitated with the lysosome associated membrane protein 1 (LAMP1), and that aSOCE is accompanied by increased cell-surface expression levels of NTCC and LAMP1 proteins. Moreover, we demonstrated that siRNA knockdown of LAMP1 or Rab27a, both of which are key proteins involved in lysosome exocytosis, attenuated significantly aSOCE. Taken together our data suggest that aSOCE occurs in neurons, that aSOCE plays an important role in regulating the levels and actions of intraneuronal calcium, and that aSOCE is regulated at least in part by exocytotic insertion of N-type calcium channels into plasma membranes through LAMP1-dependent lysosome exocytosis.

Graphical Abstract

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Conflict of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Keywords

Calcium; endolysosomes; N-type calcium channels; lysosome exocytosis; GPN; NAADP; store-operated calcium entry

1. Introduction

Intracellular calcium regulates many essential functions of neurons including neurotransmitter release, excitability, synaptic plasticity, and cell viability [1]. Levels of intraneuronal calcium are very tightly regulated both temporally and spatially by various mechanisms including calcium release from intracellular stores, calcium influx across plasma membranes, and association with calcium binding proteins. Almost 30 years ago, a capacitative model of calcium entry now known as store-operated calcium entry (SOCE) was described whereby depleting endoplasmic reticulum (ER) calcium stores triggers calcium influx into many different types of cells including neurons [2]. Mechanistically, depleting ER calcium drives oligomerization and translocation of stromal interaction molecule 1 (STIM1) proteins to ER junctions close to the plasma membrane. Such STIM1 translocation induces calcium release-activated calcium modulator 1 (Orai1) channels and/or transient receptor potential (TRP) cation channels to cluster into adjacent plasma membranes thus allowing extracellular calcium entry [3].

Intracellular organelles in addition to ER help regulate levels of intracellular calcium and these include endosomes and lysosomes (collectively referred to here as endolysosomes). Endolysosomes contain calcium stores ranging in concentration from 400 to 600 μM and these stores are now known to be readily releasable and bioactive [4, 5]. Calcium levels in endolysosomes are controlled mainly by pH gradients established by two important mechanisms; vacuolar H^+ -ATPase pumps import H^+ and Ca^{2+}/H^+ exchangers drive Ca^{2+} uptake [6]. Due to their acidic luminal pH, these endolysosome calcium stores are often referred to as “acidic calcium stores” [6]. Contributions of endolysosome stores of calcium

towards neuronal calcium signaling are starting to be elucidated [7–9]. To date, four possible mechanisms for calcium release from endolysosomes have been reported. First, nicotinic acid adenine dinucleotide phosphate (NAADP) can induce calcium release from endolysosomes through activation of two-pore channels and/or members of the mucolipin subfamily of TRP channels (TRPML) [10–14]. Second, PI (3,5) P₂ and its analogue ML-SA1 can induce calcium release from endolysosomes through activation of TRPML1 channels [15]. Third, deacidification of endolysosomes by inhibiting vacuolar H⁺-ATPase with bafilomycin and by other alkaline lysosomotropic agents such as NH₄Cl and chloroquine can induce calcium release from endolysosomes [4, 16, 17] through mechanisms including activation of TRPML channels [18] or P2X₄ receptors [19]. Fourth, selective disruption of endolysosome membranes with glycyl-L-phenylalanine 2-naphthylamide (GPN) can induce calcium release from endolysosomes [5, 20].

Calcium release from endolysosomes appears to be transient and highly localized [21–23]. However, given the dynamic properties of these organelles, calcium released from endolysosomes could result in complex communications with other organellar stores of calcium as well as with plasma membrane-based calcium influx mechanisms. Currently, three models of acidic store-induced calcium signaling mechanisms have been suggested [24]. First, acidic calcium stores might communicate with ER calcium stores such that calcium released from endolysosomes can enhance ER calcium loading [25] and calcium-induced calcium release [11, 26]. Second, changes in endolysosome pH may release calcium from a subgroup of acidic calcium stores and the released calcium may affect other subgroups of acidic stores through such mechanisms as vesicular fusion of late endosomes and lysosomes [5, 24, 26]. Third, calcium released from acidic calcium stores might depolarize plasma membranes, evoke calcium-dependent currents, and stimulate calcium influx across plasma membranes [27–30].

Neurons are long-lived post-mitotic cells that possess an elaborate system of endolysosomes. Despite recent recognition that endolysosome calcium can affect neurotransmitter release, neuronal excitability and neurite outgrowth [8, 20, 31], little is known about how this calcium integrates with other physiologically important calcium signaling processes in neurons. Here, we demonstrated that calcium released from endolysosomes, as induced by three functionally distinct mechanisms, increased surface expression of N-type calcium channels (NTCCs) that allows calcium influx across the plasma membrane. We have termed this mechanism ‘acidic store-operated calcium entry (aSOCE)’.

2. Material and Methods

2.1. Primary neuronal cultures

Primary cultures of hippocampal neurons were prepared from embryonic day 18 Sprague-Dawley rats as described previously [32] using a protocol approved by the University of North Dakota Animal Care and Use Committee adherent with the Guide for the Care and Use of Laboratory Animals (NIH publication number 80-23). Pregnant dams (embryonic day 18) were sacrificed by asphyxiation with CO₂. The fetuses were removed, decapitated, and meninges-free hippocampi were isolated, trypsinized, and plated onto poly-D-lysine-

coated glass-bottom tissue culture dishes. Neurons were grown in Neurobasal™ medium with L-glutamine, antibiotic/antimycotic and B₂₇ supplement, and were maintained at 37°C and 5% CO₂ for 10–14 days at which time they were used for experimentation. Typically, the purity of the neuronal cultures was greater than 95% as determined by neuronal immunostaining with mouse anti-NeuN or goat anti-microtubule-associated protein 2 (MAP2) antibodies (Millipore), and for astrocytes with a mouse anti-glial fibrillary acidic protein (GFAP) antibody (Sigma).

2.2. Calcium imaging

Free intraneuronal Ca²⁺ was determined using the Ca²⁺-specific fluorescent probe fura-2/AM (Invitrogen). The protocol was described and modified from our previous study [33]. Neurons were incubated with 2 μM fura-2/AM for 30 min at 37°C. Neurons were excited at 340 and 380 nm, and emission was determined at 510 nm with our filter-based calcium imaging system (Zeiss, Germany) right after being washed with different calcium buffers (as listed below) to remove extracellular fura-2. Images were acquired every 2 s and at this acquisition rate we were able to measure baseline as well as peak increases in levels of free intraneuronal calcium. The ratio of 340/380 was used as a measurement of free intraneuronal calcium levels. The following calcium buffers were used; Locke's buffer (154 mM NaCl, 3.6 mM NaHCO₃, 5.6 mM KCl, 2.3 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES and 10 mM D-glucose, pH=7.4), calcium-free buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 1 mM EGTA and 10 mM HEPES, pH=7.4), and nominal calcium buffer (154 mM NaCl, 3.6 mM NaHCO₃, 5.6 mM KCl, 1 mM MgCl₂, 5 mM HEPES and 10 mM D-glucose, pH=7.4).

2.3. Live cell imaging

To assess the ability of GPN to disrupt lysosome integrity, neurons were incubated with 100 nM LysoTracker Red (Invitrogen) or Lucifer Yellow (100 μg/ml, Invitrogen) for 30 min and stimulated with 2 μM GPN (Santa Cruz Biotech). Images were taken using confocal microscopy (Olympus).

2.4. Biotinylation of surface protein assay

Primary cultured neurons grown in 60-mm dishes were washed with nominal calcium-free buffer 3-times and treated with bafilomycin (100 nM), GPN (2 μM) or thapsigargin (TG, 2 μM) for 10 min; DMSO (0.1%) treatment served as the control. Following the treatments, CaCl₂ at a final concentration of 1 mM was added. 10 min later, neurons were incubated with sulfo-NHS-SS-biotin (0.5 mg/ml, 4°C, 30 min, Pierce) and washed with a quenching buffer (20 mM Tris and 120 mM NaCl, pH=7.4) 3-times to remove unreacted biotin. After washing twice with ice-cold PBS, neurons were lysed with 200 μl of radioimmunoprecipitation assay (RIPA) buffer containing proteinase inhibitor cocktail (Sigma) followed by sonication. After centrifugation (10,000 × g for 2 min), supernatants were collected, and protein concentrations were determined with a DC protein assay (BioRad). Equal amounts of protein were transferred to new sets of tubes, and 100 μl streptavidin-agarose beads (Pierce) were added and incubated for 16 h at 4°C. The biotin-streptavidin-agarose complexes were washed with RIPA buffer 5-times and collected by centrifugation (14,000 × g, 30 s, 4°C). The biotin-streptavidin-agarose complexes were

suspended in 1 x Laemmli buffer (containing 50 mM dithiothreitol and 5% beta-mercaptoethanol) and heated at 95°C for 10 min before being taken for immunoblotting.

2.5. Immunoprecipitation

Samples for immunoprecipitation were prepared by modifying the previous protocol [34]. Neurons were lysed with 200 µl of ice-cold RIPA buffer (50 mM Tris, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, and 50 mM NaF) containing proteinase inhibitors (Sigma). After centrifugation (14,000 × g for 10 min at 4°C), supernatants were collected, and protein concentrations were determined with a DC protein assay (Bio-Rad). After pre-clearing with protein A/G, equal amounts of protein from individual samples (400 µg) were incubated (4°C overnight) with 5 µl of antibodies against target proteins including N-type calcium channels (mouse monoclonal, Santa Cruz) or LAMP1 (rabbit polyclonal, Sigma) with irrelevant antibodies serving as controls. The immune complexes were incubated (4°C, 4 h) with 100 µl of protein A/G (Santa Cruz) while being rotated on a platform shaker. The immunoprecipitated complexes were washed with RIPA buffer 4-times, collected by centrifugation (14,000 × g for 30 s at 4°C), resuspended in 120 µl 1 x Laemmli buffer (containing 50 mM dithiothreitol and 5% beta-mercaptoethanol), and heated at 95°C for 10 min before being further analyzed with immunoblotting.

2.6. RNA interference

Targeted proteins were knocked down with specific siRNAs at a final concentration of 60 nM (Invitrogen); negative control siRNAs (Invitrogen) were used as controls. Before siRNA transfection, fresh Neurobasal™ media was added to cultured neurons that were plated for 10 days. The transfection cocktail containing 300 µl of transfection buffer (SignaGen), 12 µl of siRNA stock (15 µM) for each target protein, and 9 µl of GenMute™ reagent was added carefully to each dish along with 1 ml of media. After incubation (37°C, 5% CO₂) for 5 h, the transfection media was replaced with fresh Neurobasal™ media and further incubated for another 48 h before being taken for calcium imaging. Knockdown efficiency for each targeted protein was measured by immunoblotting. The following siRNA target sequences were used; rat STIM1 (5'-CAGUGAGAAGAAUACAGGA-3'), rat NTCC (Cacna1b, 5'-CAGUCGUUCCAGUAUAAGA-3'), rat LAMP1 (5'-GGGUAGAAAGUGACAGGUU-3'), and rat Rab27a (5'-GUUCCUCA AUGUCCGAAA-3').

2.7. Immunoblotting

The protocol was described before [35–37]. Equal amounts of proteins (10 µg) or equal volumes of samples (15 µl, for biotinylation of surface protein assay or immunoprecipitation assay) were separated by SDS-PAGE (10% gel) and then transferred to polyvinylidene difluoride membranes (Millipore), membranes were incubated overnight at 4°C, treated with primary antibodies against N-type calcium channels (1: 200, rabbit polyclonal, Millipore), N-type calcium channels (1:500, mouse monoclonal, Santa Cruz), LAMP1 (1:1000, rabbit polyclonal, Sigma), Na⁺/K⁺ ATPase (1:1000, mouse monoclonal, Millipore), and Rab27a (1:1000, rabbit polyclonal, Abcam). β-actin (1: 10,000, mouse monoclonal, Abcam) was used as a loading control. The immunoblots were developed with enhanced

chemiluminescence, and bands were visualized and analyzed by LabWorks 4.5 software on a UVP Bioimaging System (Upland). Quantification of results was performed by densitometry. Densitometric intensities of target proteins as well as loading controls (actin, Na/K ATPase or IgG) were quantified with LabWorks software. The optical density ratios of target proteins vs. loading controls were calculated and results were normalized to control values of 1.

2.8. Statistics

All data were expressed as means and SEM. Statistical significance between two groups was analyzed with a Student's t-test, and statistical significance among multiple groups was analyzed with one-way ANOVA plus a Tukey post-hoc test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Endolysosomes contain calcium that is readily releasable

To examine the extent to which endolysosomes in primary cultured neurons contain readily releasable stores of calcium capable of regulating levels of free intraneuronal calcium we used three approaches known to selectively induce calcium release from endolysosomes. First, we de-acidified endolysosome pH with either the selective vacuolar H^+ -ATPase inhibitor bafilomycin (BAF) or separately the alkaline lysosomotropic agents NH_4Cl and chloroquine [4, 16, 17]. Second, we mobilized endolysosome calcium with the endolysosome calcium-releasing agent NAADP that activates two-pore channels and/or TRPML channels [10–14]. Third, we selectively disrupted endolysosome membranes with GPN [5, 20].

In determining the contribution of endolysosome calcium stores to overall levels of free intraneuronal calcium it was important to first exclude the possible involvement of ER calcium stores. Accordingly, under calcium free conditions we treated neurons with the selective SERCA inhibitor TG (2 μM), which as expected induced a transient elevation of intracellular calcium (Figure 1A). After depleting ER calcium stores with TG we still observed release of calcium from endolysosomes (Lys) and increases in levels of intraneuronal calcium following selective inhibition of vacuolar H^+ -ATPase with 100 nM BAF (Figure 1A), selective disruption of endolysosome membranes (as indicated with LysoTracker in Figure 1E) with 2 μM GPN (Figure 1B), or activation of two pore channels and/or TRPML channels with 500 nM NAADP-AM (Figure 1C), a cell membrane permeable form of NAADP [38].

3.2. Calcium released from endolysosomes triggers calcium influx across plasma membranes

When similar experiments were conducted in the presence of extracellular calcium, we observed much larger increases in levels of intraneuronal calcium with BAF (Figure 1D), GPN (Figure 1E) and NAADP-AM (Figure 1F), and these calcium signals are comparable to those evoked by KCl-induced depolarization (Figure 1G). To determine the ability of GPN to disrupt endolysosome membrane, we used LysoTracker and Lucifer Yellow with live cell

imaging methods. We found that control cells exhibited clearly punctate fluorescence staining in the perinuclear regions and no fluorescence in the cytoplasm. In contrast, we observed lysosomal membrane leakage as indicated by diffuse fluorescence in GPN treated cells (Figure 1E). These observations suggested to us that endolysosome calcium release affects calcium influx across plasma membranes; a finding consistent with previous reports using other cell types that calcium released from endolysosomes activates cell surface calcium channels and stimulates calcium influx [27–30, 39]. To characterize mechanisms by which release of calcium from endolysosomes causes increased calcium influx, we first confirmed the functional presence of store-operated calcium entry (SOCE) involving ER. As expected, under nominal extracellular calcium conditions (no added calcium) selective inhibition of SERCA with TG (2 μ M) induced a small and transient rise in levels of free intracellular calcium. When extracellular calcium was re-introduced to the media a much larger and very rapid rise in levels of free intracellular calcium was observed (Figure 2A).

Using a similar experimental approach, we determined next the extent to which BAF, GPN, and NAADP-AM affected calcium influx. We found that under nominal extracellular calcium conditions (no added calcium), selective inhibition of vacuolar H⁺-ATPase with BAF induced a small and transient rise in levels of free intracellular calcium. However, when extracellular calcium was re-introduced to the media a much larger and very rapid rise in levels of free intracellular calcium was observed (Figure 2B). A very similar profile of calcium release and entry was observed when endolysosome membranes were selectively disrupted with GPN (Figure 2C) or endolysosome channels were activated with NAADP-AM (Figure 2D). Thus, three functionally distinct endolysosome-targeted mechanisms triggered calcium influx across plasma membranes.

Previously, it was shown that calcium released from endolysosomes could trigger calcium-induced calcium release from ER [11, 26]. Thus, it was important for us to test the extent to which our observed aSOCE might be secondary to ER-dependent store-operated calcium entry whereby calcium released from endolysosomes would trigger calcium release from ER and then trigger classical store-operated calcium entry. To test this we blocked ER-based ryanodine receptors with dantrolene (10 μ M) and then retested the ability of BAF and GPN to induce calcium influx. Blocking ryanodine receptors with dantrolene did not affect BAF- nor GPN-induced calcium influx (Figure 2E, 2F). These findings suggest that neuronal aSOCE operates separately from ER-based calcium release mechanisms.

3.3. N-type calcium channels contribute to BAF- and GPN-induced calcium influx

To characterize molecular mechanisms underlying our observations of aSOCE in neurons, we began by using a spectrum of inhibitors (listed below in parentheses) of cell surface calcium channels including ER-mediated store-operated calcium entry [SKF-96365, 2-aminoethoxydiphenylborane (2-APB)], L-type calcium channels (verapamil, nimodipine), P/Q-type calcium channels (ω -agatoxin-IVA), and N-type calcium channels (ω -conotoxin-GVIA). Of the calcium blockers tested, only the N-type calcium channel blocker ω -conotoxin attenuated significantly BAF-induced calcium influx (Figure 3A) and GPN-induced calcium influx (Figure 3B). The specificity of this effect to the exclusion of possible involvement of ER calcium stores was confirmed by showing that ω -conotoxin did not

affect TG-induced store-operated calcium entry (Figure 3C) but that SOCE was present because SKF-96365 and 2-APB both attenuated significantly TG-induced calcium influx (Figure 3C). These pharmacological findings suggested an involvement of N-type calcium channels (NTCCs) in aSOCE, and further that ER calcium stores do not play a major role in aSOCE. However, because both SKF-96365 and 2-APB are nonselective reagents with multiple actions in addition to their effects on ER calcium stores, our pharmacological findings could not exclude the involvement of ER calcium stores in aSOCE. To further exclude the possible involvement of ER calcium stores in aSOCE, we knocked down the protein expression levels of STIM1, a key protein that is responsible for ER-mediated SOCE. We found that when levels of STIM1 were knocked down by about 50% with siRNA (Figure 3D), TG-induced calcium influx was attenuated significantly (Figure 3E), but no significant effects were noted for BAF-induced (Figure 3F) or GPN-induced (Figure 3G) calcium influx.

To extend our pharmacological findings that NTCCs are involved in aSOCE, we determined next the extent to which siRNA knockdown of NTCCs affected aSOCE. The siRNA treatment decreased significantly protein expression levels of NTCCs by about 55% (Figure 4A). Further, we demonstrated that siRNA knockdown of NTCCs did not affect TG-induced ER-mediated SOCE (Figure 4B), but did attenuate significantly BAF- and GPN-induced calcium influx (Figure 4C, D). These findings add evidence that NTCCs are involved with aSOCE in primary cultured neurons.

3.4. aSOCE and increased cell surface expression levels of NTCCs

To further investigate possible mechanisms whereby BAF and GPN can activate aSOCE, we determined neuronal surface expression levels of NTCCs and LAMP1. By quantitative biotinylation of surface proteins we found that BAF and GPN, but not TG, increased significantly ($p < 0.05$) cell surface protein levels of NTCCs and LAMP1 (Figure 5A). These increases in cell surface expression levels were highly localized because neither BAF nor GPN affected protein levels of NTCCs and LAMP1 in total cell extracts (Figure 5A).

3.5. NTCCs interact physically with LAMP1

Our observations that increased cell surface expression levels of both NTCCs and LAMP1 were associated with BAF- and GPN-induced aSOCE promoted us to investigate possible physical interactions between NTCCs and LAMP1. Using co-immunoprecipitation methods we showed that BAF and GPN, but not TG, increased protein levels of NTCCs when immunoprecipitated with LAMP1 antibodies (Figure 5B). Conversely, when immunoprecipitated with NTCC antibodies, BAF and GPN, but not TG, increased protein levels of LAMP1 (Figure 5C). These findings indicate physical interactions between NTCCs and LAMP1. Because RIPA buffer containing SDS was used in our immunoprecipitation studies, interactions between NTCCs and LAMP1 were obviously strong. Our findings are consistent with reports that NTCCs are present in lysosomes [40] and that LAMP1 can translocate to plasma membranes [41].

3.6. Role of LAMP1-dependent lysosome exocytosis in aSOCE

Lysosome exocytosis is a calcium-regulated process that can insert calcium channels into neuronal plasma membranes where they can participate in membrane repair, neurite outgrowth and nociception [42–46]. Because LAMP1 is critical for lysosome exocytosis [31, 47] and because we found that NTCCs are physically associated with LAMP1, we determined next the extent to which enhanced calcium influx was mediated through LAMP1-dependent lysosome exocytosis. Accordingly, we knocked down protein levels of LAMP1 with siRNA (Figure 6A) and found that when expression levels of LAMP1 were reduced there was a statistically significant attenuation in BAF-induced (Figure 6B) and GPN-induced (Figure 6C) calcium influx. Furthermore, because Rab27a, a small G protein that interacts with LAMP1 [41], serves as an essential regulator in lysosome exocytosis [48–50] we knocked down protein expression levels of Rab27a (Figure 6D) and again found significant ($p < 0.05$) decreases in BAF-induced (Figure 6E) and GPN-induced (Figure 6F) calcium influx. These results suggest an important role of lysosome exocytosis in aSOCE.

4. Discussion

Acidic store-operated calcium entry (aSOCE) is a unique mechanism that links readily releasable calcium in endolysosomes with influx of extracellular calcium into neurons. This is a novel means by which intraneuronal stores of calcium can contribute to spatial and temporal integration of calcium signaling. In support of this novel mechanism we found that calcium could be released from endolysosomes following stimulation of a number of different mechanisms, that the calcium release was independent of other organellar stores of calcium, that release of calcium from endolysosomes triggered calcium influx, that the calcium influx was regulated by NTCCs, that NTCCs interacted physically with the lysosome marker protein LAMP1, and that LAMP1-dependent lysosome exocytosis could insert NTCCs into plasma membranes where they participated in aSOCE.

Neuronal calcium signals display spectacular spatiotemporal complexity, and understanding how calcium signals are generated spatially and temporally is necessary to understand calcium-dependent cellular processes. Over the last 15 years, endolysosomes were shown to contain calcium levels of hundreds of micromolar and that the calcium is readily releasable [4, 5, 23]. Given the dynamic properties of these organelles, calcium released from these acidic stores can regulate a variety of fundamental calcium-dependent processes including vesicular trafficking, endolysosome fusion, lysosome-related organelle biogenesis, membrane repair, and exocytosis-endocytosis coupling [26, 51–55]. Indeed, for neurons, endolysosome calcium has been implicated in neurotransmitter release, neuronal excitability, synaptic plasticity, neurite extension, and neuronal viability [7–9, 56]. However, little is known about how endolysosome calcium contributes to the spatiotemporal complexity of calcium signaling in neurons.

Here, we demonstrated the presence of readily releasable calcium from endolysosomes in primary cultured neurons. This was supported by findings with three known agents that selectively induce calcium release from endolysosomes via different mechanisms; increases in intracellular calcium under calcium-free conditions were shown following inhibition of vacuolar H^+ -ATPase with BAF, selective disruption of endolysosome membranes with

GPN, and activation of two-pore and/or TRPML channels with NAADP-AM. Furthermore, we found that all three of these agents selectively induced large amounts of calcium influx. In some respects, this aSOCE resembles TG-induced ER-dependent store-operated calcium entry (SOCE), where depleting ER calcium with TG results in STIM1 oligomerization and translocation to ER junctions close to the plasma membrane followed by induction of Orai1 and/or TRP channels to cluster in the adjacent plasma membrane thus allowing large amount of calcium influx [3, 57]. Although we do not understand completely why a small release of calcium from endolysosomes triggers such a large calcium influx, plasma membrane depolarization as is accompanied by NAADP-induced endolysosome calcium release might be the reason [27, 39]. Indeed NAADP-induced endolysosome calcium release can affect calcium influx at the plasma membrane in cells other than neurons, a phenomena similar to our observed aSOCE [27–30, 58]; however, very little is known about such mechanisms in neurons.

Taken together our data support the notion that aSOCE is a novel calcium influx mechanism distinct from ER-based SOCE. This view is supported by our findings that ER-dependent store-operated calcium channel blockers (2-APB and SKF) did not affect BAF- or GPN-induced calcium influx, but did block ER-mediated SOCE, and that blocking calcium-induced calcium release from ER did not affect BAF- nor GPN-induced calcium influx. In addition, siRNA knockdown of STIM1, the key protein responsible for SOCE, attenuated significantly TG-induced calcium influx, but did not affect BAF- or GPN-induced calcium influx. Moreover, pharmacological inhibition of NTCCs or siRNA knockdown of NTCCs attenuated significantly BAF- and GPN-induced calcium influx, but did not affect ER-mediated SOCE. Together, our findings suggest that instead of secondary activation of classical SOCE, NTCCs are responsible for our observations of aSOCE in cultured neurons.

In addition to ER-mediated SOCE, two other possible mechanisms might be considered to underlie aSOCE; calcium might be released from endolysosomes close to plasma membranes to open calcium-activated ion channels [24, 27, 58, 59] or calcium might trigger exocytotic insertion of calcium channels into plasma membranes. Our findings support the notion that exocytotic insertion of NTCCs is central to the mechanism of aSOCE. Lysosomes can behave as calcium-regulated exocytotic vesicles during lysosome exocytosis. Indeed, localized elevations of calcium can trigger lysosome exocytosis [16, 17] and insert calcium channels into plasma membrane whereby they might contribute to membrane repair, compensatory endocytosis at the sites of exocytosis, neurite outgrowth, and nociception [42–46]. It has been shown that regulated exocytosis could insert functionally intact NTCCs into plasma membranes [60]. Given findings that NTCCs are present in lysosomes [40], we next performed studies to determine the extent to which exocytotic insertion of NTCCs are mechanistically linked to aSOCE. We showed using surface immunostaining and quantitative biotinylation of surface protein assays, that BAF and GPN, but not TG, increased surface expression levels of NTCCs and LAMP1, which is a lysosome associated membrane protein that is critical for lysosome exocytosis [47]. Thus, our studies favor the involvement of exocytotic insertion of NTCCs in aSOCE.

To further determine the underlying molecular mechanisms of aSOCE, we then tested the possible interaction between NTCCs and LAMP1, and our co-immunoprecipitation findings

indicated a physical interaction between NTCCs and LAMP1; a finding consistent with a report that NTCCs are present in lysosomes [40]. Such findings support our hypothesis that LAMP1-dependent lysosome exocytosis can insert NTCCs into plasma membranes and facilitate calcium influx via aSOCE. To further strengthen this idea we knocked down protein expression levels of LAMP-1 with siRNA strategies and found that this treatment attenuated significantly BAF- or GPN-induced calcium influx, but not TG-induced calcium influx. Furthermore, we knocked down protein expression levels of Rab27a, another key protein involved in lysosome exocytosis [48–50], and again observed a significant attenuation of BAF- and GPN-induced calcium influx. Thus, our findings support the notion that LAMP1-dependent lysosome exocytosis can insert NTCCs into plasma membranes during BAF- and GPN-induced aSOCE. Given findings that calcium release from endolysosomes could depolarize plasma membrane [27], the newly inserted NTCCs could be activated thus promoting calcium influx in neurons. In our studies, calcium influx as induced by BAF or GPN is most likely relevant to pathological insults and not physiological conditions. The physiological significance of lysosome exocytotic insertion of NTCCs into plasma membranes is not known. However, we speculate that such rapid and presumably localized insertion of NTCCs into plasma membranes resulting from lysosome exocytosis may help regulate exocytosis-endocytosis coupling. Indeed, calcium Influx is known to affect endocytosis [61] and further mechanistic studies are warranted to determine the role of aSOCE in endocytosis and other physiological processes.

Our demonstration of a novel mechanism (Figure 7) in neurons whereby endolysosome calcium release triggers calcium influx into neurons - a mechanism we have termed “acidic store-operated calcium entry (aSOCE)” - provides a new insight into the spatiotemporal complexity of neuronal calcium signaling and the involvement of endolysosomes.

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Highlights

- Calcium released from endolysosomes triggers calcium influx across plasma membranes, a process we have termed acidic store operated calcium entry (aSOCE).
- aSOCE is a process independent of endoplasmic reticulum calcium release.
- aSOCE is mediated at least in part by N-type calcium channels.
- N-type calcium channels interacts with lysosome marker protein LAMP1 and knocking down LAMP1 attenuated aSOCE.

Figure 1. Calcium stores in endolysosomes contributed to levels of free intraneuronal calcium (A,B,C) Under calcium-free conditions (A) selective inhibition of vacuolar H^+ -ATPase with bafilomycin (BAF 100 nM), (B) selective disruption of endolysosome membranes with glycyl-L-phenylalanine 2-naphthylamide (GPN 2 μ M) or (C) selective activation of two pore channels with nicotinic acid adenine dinucleotide phosphate acetoxymethyl ester (NAADP-AM 500 nM) each induced calcium release from neuronal endolysosome compartments (Lys) after first releasing calcium from ER calcium stores with the selective SERCA inhibitor thapsigargin (TG, 2 μ M). (D,E,F) Under calcium-free conditions, BAF (100 nM), GPN (2 μ M), and NAADP-AM (500 nM) each induced a small and transient rise in levels of intracellular calcium (grey line). By contrast, in the presence of 1 mM extracellular calcium BAF, GPN and NAADP-AM increased markedly levels of intracellular calcium (black line). Using LysoTracker and Lucifer Yellow to determine endolysosome integrity, we demonstrated that control cells exhibit clear punctate fluorescence staining in the perinuclear regions and with no fluorescence in the cytoplasm, whereas lysosomal membrane leakage as indicated by diffuse fluorescence in GPN treated cells (E). (G) KCl (50 mM) increased markedly levels of intracellular calcium in the presence of 1 mM extracellular calcium.

Figure 2. Calcium released from endolysosomes triggered calcium influx across neuronal plasma membrane

(A) Typical of ER-dependent store-operated calcium entry, thapsigargin (TG, 2 μ M) induced a small and transient rise in levels of intracellular calcium under nominally calcium-free conditions and this was followed by a dramatic increase in calcium influx when calcium (1 mM) was re-added to the media. (B) BAF (100 nM) induced a small and transient rise in intracellular calcium under nominally calcium-free conditions, and this was followed by a dramatic increase in calcium influx when calcium (1 mM) was re-added to the media. (C) GPN (2 μ M) induced a small and transient rise in intracellular calcium under nominally calcium-free conditions, and this was followed by a dramatic increase in calcium influx when calcium (1 mM) was re-added to the media. (D) NAADP-AM (500 nM) induced a

small and transient rise in intracellular calcium under nominally calcium-free (zero calcium, no EGTA added) conditions, and this was followed by a dramatic increase in calcium influx when calcium (1 mM) was re-added to the media. (E) Pretreatment neurons with a ryanodine receptor blocker dantrolene (10 μ M for 30 min) did not affect BAF-induced calcium influx (n=11, p>0.05). (F) Pretreatment neurons with a ryanodine receptor blocker dantrolene (10 μ M for 30 min) did not affect GPN-induced calcium influx (n=8, p>0.05).

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Figure 3. N-Type calcium channels (NTCCs) mediated BAF- and GPN-induced calcium influx (A) The NTCC blocker, ω -conotoxin attenuated significantly BAF-induced calcium influx ($n=15$, *** $p<0.001$); no significant decreases in calcium influx was observed with the other calcium channel blockers including L-type calcium channel blockers (nimodipine 10 μ M or verapamil 10 μ M), P/Q-type calcium channel blocker (ω -agatoxin 10 μ M), and ER-dependent store-operated calcium channel blockers (2-APB 100 μ M or SKF 50 μ M), (B) Only the NTCC blocker, ω -conotoxin, attenuated significantly GPN-induced calcium influx ($n=15$, *** $p<0.001$). (C) As a control, the store-operated calcium channel blockers, 2-APB (100 μ M) and SKF (50 μ M), both attenuated significantly thapsigargin (TG)-induced calcium influx ($n=18$, *** $p < 0.001$). (D) STIM1 siRNA (60 nM) treatment decreased significantly protein levels of STIM when compared with negative control siRNA ($n = 4$, * $p < 0.05$). (E) siRNA knockdown of STIM1 decreased significantly TG-induced calcium influx ($n = 32$, *** $p < 0.001$); negative control siRNA treatment did not affect TG-induced calcium influx ($n = 24$, $p > 0.05$). (F) siRNA knockdown of STIM1 did not affect significantly BAF-induced calcium influx ($n = 13$, $p > 0.05$); negative control siRNA treatment did not affect BAF-induced calcium influx. (G) siRNA knockdown of STIM1 did

not affect significantly GPN-induced calcium influx ($n = 20$, $p > 0.05$); negative control siRNA treatment did not affect GPN-induced calcium influx.

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Figure 4. NTCCs were essential for BAF- and GPN-induced calcium influx

(A) NTCC siRNA (60 nM) treatment decreased significantly protein levels of NTCCs when compared with negative control siRNA ($n = 4$, $*p < 0.05$). (B) Neither siRNA knockdown of NTCCs or negative control siRNA treatment affected TG-induced calcium influx ($n = 35$, $p > 0.05$). (C) siRNA knockdown of NTCCs decreased significantly BAF-induced calcium influx ($n = 66$, $***p < 0.001$); negative control siRNA treatment did not affect BAF-induced calcium influx. (D) siRNA knockdown of NTCCs decreased significantly GPN-induced calcium influx ($n = 59$, $***p < 0.001$); negative control siRNA treatment did not affect GPN-induced calcium influx.

Figure 5. BAF and GPN increased cell surface expression levels of NTCCs and LAMP1, and NTCCs interact physically with LAMP1

(A) Biotinylated surface protein assays showed that bafilomycin (BAF, 100nM) and glycyl-L-phenylalanine 2-naphthylamide (GPN, 2 μ M), but not by thapsigargin (TG, 2 μ M) increased significantly surface protein levels of NTCCs and LAMP1 ($n = 5$, $*p < 0.05$). Neither BAF nor GPN affected protein levels of NTCCs or LAMP1 in whole cell lysates. β -actin was used as a control and Na⁺/K⁺ ATPase was used to identify plasma membrane components of primary cultured neurons. (C) Immunoprecipitation with LAMP1 antibodies showed that BAF and GPN, but not TG, increased physical interactions between LAMP1 and NTCCs ($n = 4$, $*p < 0.05$). (D) Immunoprecipitation with NTCC antibodies showed that

BAF and GPN, but not TG, increased physical interaction between NTCCs and LAMP1 (n = 4, *p < 0.05).

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Figure 6. LAMP1 and Rab27a mediated BAF- and GPN-Induced calcium influx

(A) LAMP1 siRNA treatment decreased significantly protein levels of LAMP1, when compared with negative control siRNA (n = 6, $p < 0.05$). (B) siRNA knockdown of LAMP1 decreased significantly BAF-induced calcium influx (n=53, *** $p < 0.001$). (C) siRNA knockdown of LAMP1 decreased significantly GPN-induced calcium influx (n=22, *** $p < 0.001$). (D) Rab27a siRNA treatment decreased significantly protein levels of Rab27a, when compared with negative control siRNA (n = 4, * $p < 0.05$). (E) siRNA knockdown of Rab27a decreased significantly BAF-induced calcium influx (n=18, ** $p < 0.01$). (F) siRNA knockdown of Rab27a decreased significantly GPN-induced calcium influx (n=22, ** $p < 0.01$).

Figure 7. Molecular mechanism of acidic store-operated calcium entry (aSOCE)

(A) According to the widely accepted model for store-operated calcium entry (SOCE), upon depletion of ER calcium stores by blocking sarco/endoplasmic reticulum calcium ATPase with thapsigargin (TG), STIM1 oligomerizes and moves to ER-plasma membrane junctions where it opens Orai1 or TRP channels to allow calcium influx. (B) In neurons, calcium released from endolysosomes (acidic calcium stores) triggers calcium influx across the plasma membrane, a phenomenon we termed acidic store-operated calcium entry (aSOCE). In this model, calcium release from acidic calcium stores triggers LAMP1-dependent lysosome exocytosis. Because of physical interactions between LAMP1 and N-type calcium channels (NTCCs), such lysosome exocytosis promotes the insertion of NTCCs into plasma membranes thus allowing calcium influx across plasma membranes through NTCCs.