A voltage-dependent K⁺ channel in the lysosome is required for refilling lysosomal Ca²⁺ stores

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The resting membrane potential (Δψ) of the cell is negative on the cytosolic side and determined primarily by the plasma membrane’s selective permeability to K⁺. We show that lysosomal Δψ is set by lysosomal membrane permeabilities to Na⁺ and H⁺, but not K⁺, and is positive on the cytosolic side. An increase in juxta-lysosomal Ca²⁺ rapidly reversed lysosomal Δψ by activating a large voltage-dependent and K⁺-selective conductance (LysoKVCa). LysoKVCa is encoded molecularly by SLO1 proteins known for forming plasma membrane BK channels. Opening of single LysoKVCa channels is sufficient to cause the rapid, striking changes in lysosomal Δψ. Lysosomal Ca²⁺ stores may be refilled from endoplasmic reticulum (ER) Ca²⁺ via ER–lysosome membrane contact sites. We propose that LysoKVCa serves as the perilysosomal Ca²⁺ effector to prime lysosomes for the refilling process. Consistently, genetic ablation or pharmacological inhibition of LysoKVCa, or abolition of its Ca²⁺ sensitivity, blocks refilling and maintenance of lysosomal Ca²⁺ stores, resulting in lysosomal cholesterol accumulation and a lysosome storage phenotype.

Introduction

The precise delivery of hydrolases and cargo to lysosomes for degradation and the timely removal of lysosomal catabolites require the establishment of luminal ionic homeostasis, ionic membrane gradients, and a membrane potential (Δψ; Mindell, 2012; Xu and Ren, 2015). The lysosomal membrane maintains 1,000- to 5,000-fold concentration gradients for Na⁺ and Ca²⁺ (Xu and Ren, 2015). It has been established that lysosomal H⁺ homeostasis is required for hydrolase activation (Mindell, 2012) and that lysosomal Ca²⁺ efflux mediates signals integral to lysosomal membrane trafficking; however, the lysosomal effectors on which Ca²⁺ acts are largely unknown (Kiselyov et al., 2010; Shen et al., 2012). Several specific ion-dependent channels/transporters have been identified in lysosomes, including the V-ATPase H⁺ pump and transient receptor potential mucolipin channels (TRPMLs), the principle Ca²⁺ release channels in the lysosome (Medina et al., 2015; Wang et al., 2015; Xu and Ren, 2015). H⁺ channels and Ca²⁺ transporters in the lysosomes, however, remain to be molecularly identified (Xu and Ren, 2015; Garrity et al., 2016).

Much less is understood about the roles of Na⁺ and K⁺ in lysosomal physiology. Although manipulations of lysosomal Na⁺ and K⁺ with ionophores can affect several lysosomal functions (Morgan et al., 2011), it was not recognized until recently that, based on ionic composition analysis of isolated lysosomes, there may exist large concentration gradients (>10-fold) across lysosomal membranes for both ions ([Na⁺]lumen >> [Na⁺]cytosol, ENa > 57 mV; [K⁺]lumen << [K⁺]cytosol, EK < ~57 mV; Wang et al., 2012; Xu and Ren, 2015). With these ion concentration gradients, and based on knowledge extrapolated from plasma membrane studies, resting lysosomal Δψ has been expected to be set by the membrane’s relative permeability to K⁺ over Na⁺ ions (PK/PNa; Cang et al., 2015; Xu and Ren, 2015). Lysosomal Δψ, considered essential for lysosome function (Mindell, 2012), is thought to be negative (VLumen = 30 mV; VCytosol is defined to 0 mV; Δψ = VLumen − VCytosol = −VLumen ~ −30 mV), which is suggestive of high PK/PNa in rest conditions. Considering that lysosomal H⁺ permeability may also contribute a positive value to lysosomal Δψ, background Pk at resting conditions is expected to be even higher to maintain a negative lysosomal Δψ. Note that depending on the ratio of [Cl⁻]lumen vs. [Cl⁻]cytosol, background PCl would also contribute a small positive or negative value to lysosomal Δψ (Mindell, 2012).

Recently, we identified two-pore channels (TPCs) as the major Na⁺-selective channels in the lysosome that affect Δψ (Wang et al., 2012). Although still controversial, TPCs may also mediate lysosomal Ca²⁺ or H⁺ conductance (Patel, 2015). The
main goal of the current study was to identify the lysosomal K⁺ channels that regulate lysosomal ΔΨ (Xu and Ren, 2015). During the course of this study, two other research groups reported the existence of K⁺-selective channels in the lysosome (Cang et al., 2015; Cao et al., 2015). The functional identification of Na⁺- and K⁺-selective channels in the lysosome is consistent with the notion that the lysosome lumen is a high Na⁺ but low K⁺ compartment (Wang et al., 2012; Xu and Ren, 2015). However, our voltage- and current-clamping analyses of isolated lysosomes in the current study suggest that in contrast to previous views, lysosomal ΔΨ is positive, and background P_K is minimal.

Results

Ubiquitous Ca²⁺-activated K⁺-current in the lysosomes, but not early endosomes

To investigate lysosomal K⁺-selective conductances, we performed whole-endolysosome recordings using a high K⁺ bath/cyttoplasmic solution ([K⁺]_C = 140 mM; [K⁺]_L = 5 mM; pH_Ly = 4.6; Fig. 1 A) in various mammalian cell lines and primary cells. In Cos-1 cells, endolysosomes were enlarged from <0.5 to ≤5 μm with vacuolin-1 (Dong et al., 2008). Vacuolin-1 selectively enlarged endosomes and lysosomes, but not other intracellular organelles such as mitochondria or ER (Cerny et al., 2004). Although EEA1-positive early endosomes were also slightly enlarged, only Lamp1-positive vacuoles were enlarged to patchable sizes (Fig. S1 A). Enlarged vacuoles were then isolated and patch-clamped (Dong et al., 2008; Wang et al., 2012). Negligible or no outward currents (<50 pA at 120 mV) were seen in most vacuoles, suggesting that the background TMEM175-like K⁺ conductance reported by Cang et al. (2015) under high luminal K⁺ and pH recording conditions is minimally active under our experimental conditions. However, increasing the concentration of Ca²⁺ from the bath/cyttoplasmic side ([Ca²⁺]_C) to 100–1,000 μM induced the rapid appearance of large (>200 and ≤3,000 pA at 120 mV) outwardly rectifying currents (Fig. 1, B and C). Similar Ca²⁺-activated outward currents were detected in endolysosomes enlarged spontaneously (Dong et al., 2010), by sucrose treatment (Bandyopadhyay et al., 2014), or by apilimod treatment (Cai et al., 2014; Fig. S1 B). In contrast, Ca²⁺-activated outward currents were not detected in isolated early endosomes, which could be artifically enlarged by either overexpression of a dominant-negative Rab5 mutant (Rab5-Q79L) in the cells (Wegner et al., 2010) or pretreatment with vicenistatin (Nishiyama et al., 2016; Fig. 1, D and E; and Fig. S1 C), suggesting the lysosome specificity of the currents.

Substitution of luminal Cl⁻ with gluconate−, which largely abolished the background outwardly rectifying Cl⁻ currents seen in some patches, did not affect the Ca²⁺-activated outward currents, suggesting that the currents were mediated by influx of K⁺ into the lysosomal side (Fig. 1 F). Consistent with this interpretation, replacement of K⁺ with Na⁺ or Cs⁺ in the bath solution abolished the currents completely (Fig. 1 G). Because current activation is also strongly dependent on membrane voltage (Fig. 1, B, F, and G), we define this Ca²⁺-activated current as the lysosomal voltage- and Ca²⁺-activated K⁺ current (LysoKVCa). Notably, LysoKVCa was noisy (Fig. 1, B, F, and G), as is characteristic of channels with high unitary conductance. LysoKVCa was detected ubiquitously in various mammalian cell types, including HEK293T cells, CV1 monkey kidney cells, A7r5 smooth muscle cells, INS-1 pancreatic cells, primary mouse cortical neurons, mouse bladder epithelial cells (BECs), mouse embryonic fibroblasts (MEFs), and mouse parietal cells (Fig. 2, A and D; and Fig. S2, A–F and J).

LysoKVCa is mediated by SLO1

LysoKVCa resembles the BK (maxi-K) currents at the cell surface of excitable cells, such as muscle cells and neurons (Shi et al., 2002; Salkoff et al., 2006; Yuan et al., 2010). BK channels are formed by the coassembly of the pore-forming SLO1 (KCNM1A1) subunit and auxiliary β (KCNM1B1–4) or γ subunits (Salkoff et al., 2006; Yuan et al., 2010). Unlike wild-type (WT) MEFs, in the KCNM1A1 knockout (KO) MEFs (Fig. S2 I), no LysoKVCa-like currents were seen (Fig. 2, A, B, and D). Likewise, LysoKVCa currents were detected in WT but not KCNM1A1 KO mouse parietal cells (Figs. 2 D and S2 J). In contrast, endogenous, background, whole-cell K⁺-selective outward currents were not different between WT and KCNM1A1 KO MEF cells (Fig. S2 K). It should be noted that the plasma membrane background K⁺ conductances (Fig. S2 K), which are known to set the resting membrane potential of the cell, were undetectable in the lysosomes of KCNM1A1 KO cells (Fig. 2, B and D; and Fig. S2 I), suggesting that BK channels are uniquely targeted to lysosomes.

On the other hand, overexpression of mouse SLO1-YFP (YFP tag is in the cytoplasmic side) or human SLO1-GFP in Cos-1 cells resulted in large LysoKVCa-like currents, even under basal conditions ([Ca²⁺]_C = 0.1 μM; Fig. 2, C and D), and those currents could be augmented further by increasing cytoplasmic Ca²⁺ (Fig. 2 C). In contrast, overexpression of other Kᵥ channels (e.g., Kᵥ2.1-GFP) failed to increase whole-endolysosomal K⁺ currents. Collectively, these results suggest that SLO1 proteins are the molecular mediators of LysoKVCa.

SLO1 has been observed in intracellular organelles, including the nucleus and mitochondria, of excitable cells in addition to the plasma membrane (Singh et al., 2012, 2013; Li et al., 2014). Organelle fractionation analysis revealed that SLO1-YFP proteins (splicing variants containing the VEDEC sequence; Singh et al., 2013) in transfected Cos-1 cells were enriched in both Lamp1-resident lysosomal fractions and complex II–resident mitochondrial fractions (Fig. 2 E). Furthermore, fluorescence analysis showed that overexpressed SLO1 proteins were localized predominantly in the Lamp1-positive late endosomal and lysosomal compartments (Fig. 2, F and G). In contrast, under the same microscopic settings, SLO1 was rarely colocalized to a significant degree with markers for nuclear membranes, early endosomes, ER, Golgi apparatus, or even the plasma membrane, although partial colocalization was also observed for mitochondrial markers (Fig. 2, F and G; and Fig. S2 L). Intriguingly, although large whole-cell BK currents can be measured (Fig. S4 G), in nonexcitable cells, plasma membrane localization of overexpressed SLO1 proteins could be detected only with the aid of surface-specific labeling (Liu et al., 2014). Collectively, these results suggest that, consistent with electrophysiological analyses, the localized, high levels of SLO1 protein expression in lysosomal membranes gives rise to LysoKVCa. Dileucine motifs could be responsible for specific targeting of SLO1 proteins to lysosomes (Cao et al., 2015). However, mutations in these motifs did not significantly decrease SLO1-mediated lysosomal (Lyso-SLO1) currents in our hands (Fig. S2, M and N).
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Dual activation of LysoKVCa by Ca2+ and voltage

A unique gating property of BK channels is their dual activation by membrane voltage and cytoplasmic Ca2+ (Salkoff et al., 2006). Whole-endolysosomal currents elicited by voltage steps revealed that increasing [Ca2+]c from basal levels (0.1 µM) to 100–1,000 µM activated outwardly rectifying currents robustly at positive voltages (Figs. 3 A and S3 A). Consistent with LysoKVCa’s K+ selectivity, the Ca2+-activated step currents had an Erev of less than −60 mV under physiology-mimicking recording conditions (calculated EK = −85.6 mV) and an Erev of ∼0 mV under symmetric K+ solutions.

In SLO1-YFP-expressing Cos-1 cells, whole-endolysosome LysoKVCa step currents were observed in basal Ca2+ level conditions at voltages that were less positive than in non-transfected cells (Figs. 3 B and S3 B). An analysis of normalized conductance–voltage curves revealed significant leftward shifts in half-maximal activation voltage (V0.5) when [Ca2+]c was increased in the range of 3–100 µM (Fig. S3, C and D). Similar to BK channels (Yang et al., 2008), cytoplasmic Mg2+ activated LysoKVCa and Lyso-SLO1 channels, but blocked the conductance (Fig. S3, F–H).

LysoKVCa exhibits a high single-channel conductance

Consistent with our whole-endolysosome recordings, in a subset of large-sized cytoplasmic-side-out endolysosomal patches, increasing [Ca2+]c induced robust large-amplitude single-channel openings of LysoKVCa (Fig. 3 C), with a half-maximal concentration (EC50) of ∼20 µM at 80 mV (Fig. S3 D). These results suggest that small changes in local juxta-lysosomal Ca2+ could readily activate LysoKVCa. Single-channel LysoKVCa currents were occasionally seen in small-sized lysosomal patches from SLO1-expressing Cos-1 cells (Fig. S3 E), which typically displayed large macroscopic currents (Fig. 3 D). At 100 µM [Ca2+]c, the channel open...
probability (P_{o_p}) of LysoK_{VCa} displayed strong voltage dependence (Figs. 3 E and S3 E). In symmetric K+ solutions, the single-channel conductances for LysoK_{VCa} and Lyso-SLO1 were 231 ± 61 and 219 ± 59 pS (n = 3), respectively (Figs. 3 F and S3 I). Hence, LysoK_{VCa} conducts K+ efficiently with a large conductance.

Pharmacological and physiological modulation of LysoK_{VCa}

Both LysoK_{VCa} and Lyso-SLO1 exhibited pharmacological properties similar to those of BK channels. Paxilline, a relatively specific membrane-permeable BK inhibitor (Salkoff et al., 2006), inhibited LysoK_{VCa} and Lyso-SLO1 completely (Figs. 3 G and S4, A and E). Quinidine and clofilium, membrane-permeable BK inhibitors with lower specificity than paxilline (Tang et al., 2010), also inhibited both LysoK_{VCa} and Lyso-SLO1 (Figs. 3 H and S4, B and E). Likewise, in the luminal-side-out patches, iberiotoxin (IBTX), a membrane-impermeable BK-specific toxin inhibitor (Tang et al., 2010), completely inhibited single LysoK_{VCa} currents (Fig. 3 I). Conversely, NS1619 (Olesen et al., 1994) and isopimaric acid (Yamamura et al., 2001), BK-specific channel openers, augmented LysoK_{VCa} (Fig. S4, C–E).

Both luminal and cytoplasmic pH are critical determinants of lysosomal physiology (Xu and Ren, 2015). Interestingly, an elevation of lysosomal pH, from 4.6 to 7.4 markedly enhanced the Ca2+ activation of Lyso-SLO1 (Fig. S4, F–H). On the other hand, acidic pH_C readily activated LysoK_{VCa} at basal Ca2+ levels (Fig. S4, I–K). These results suggest that under physiological conditions, lysosomal H+ release may activate LysoK_{VCa} by simultaneously raising luminal pH and decreasing juxta-lysosomal pH.
Regulation of LysoK_{VCa} by auxiliary subunits and trafficking

The functional diversity of BK channels can be conferred by cell type-specific or location-specific assembly of SLO1 with various auxiliary β and γ subunits (Yan and Aldrich, 2010; Hoshi et al., 2013). For example, β2 and a subset of β3 isoforms are known to confer fast and voltage-dependent inactivation of BK currents, respectively, in both heterologous expression and endogenous settings (Zeng et al., 2001; Xia et al., 2003; Torres et al., 2014). Notably, overexpression of β2-GFP together with SLO1 resulted in fast-inactivating LysoK_{VCa} currents (Fig. 4 A). In INS-1 cells, which express β3 subunits (Braun et al., 2008; Torres et al., 2014), and cultured BECs, LysoK_{VCa} exhibited voltage-dependent inactivation or blockage at high voltages (Fig. S2, D and E, and Fig. S5 A). Notably, heterologously expressed β subunits, including β2, were found to be localized in Lamp1-positive compartments (Figs. 4 B and S5 B).

We examined whether endogenous SLO1 proteins are present at the plasma membrane of LysoK_{VCa}-expressing non-excitable cells. In Cos-1 cells, measurable whole-cell paxilline- and IBTX-sensitive BK-like currents were detected (Fig. 4 C and Fig. S5, C–F), but the current density was, at most, a tenth of that of LysoK_{VCa} (Fig. 4 D). Consistently, in MEFs, no measurable whole-cell BK-like currents were detected (Figs. 4 D and S5 G). Hence, in nonexcitable cells, SLO1 proteins may be preferentially or specifically targeted to lysosomes where they mediate LysoK_{VCa}. Regulation of lysosomal membrane potential by Ca^{2+} and LysoK_{VCa}

In excitable cells, BK acts as a negative-feedback regulator of membrane excitability (Salkoff et al., 2006). To investigate the role of LysoK_{VCa} in regulating lysosomal excitability, we performed current-clamp recordings on isolated enlarged endolysosomes. Lysosomal Δψ (i.e., V_{Cytoplasm} − V_{Lumen} = −V_{Lumen}, because V_{Cytoplasm} is defined to 0 mV; Fig. 5, A and B) is thought to be negative (approximately −30 mV; Morgan et al., 2011). Under our experimental conditions (high lysosomal Na+ and low lysosomal K+; Wang et al., 2012; Fig. 5, A and B), direct electrophysiological measurement revealed that Δψ was in fact positive (15–30 mV; Fig. 5, C–F). Removal of either luminal Na+ or H+ reduced Δψ by ∼15–20 mV, whereas removal of both ions simultaneously caused a reversal of Δψ to approximately −30 mV (Fig. 5 D). Similarly, bath application of the K+ ionophore valinomycin (Van Dyke, 1995) also reversed lysosomal Δψ (Fig. 5 C), suggesting a limited K+ permeability at rest conditions and, in contrast to the plasma membrane, an increase of K+ permeability in the lysosome that would result in a large change of lysosomal Δψ. Conversely, bath application of H+ ionophores niclosamide (Fonseca et al., 2012) or activation of TPCs with PI(3,5)P_{2} (Wang et al., 2012) caused a further positive shift in Δψ (Fig. 5, C and J). In the absence of PI(3,5)P_{2}, inclusion of ATP in the bath solution had a minimal effect on lysosomal Δψ (Fig. 5 E). Together, these results suggest that in the enlarged lysosomes, the primary determinants of “resting” lysosomal Δψ are the lysosomal membrane’s Na+ and H+ per-
meabilities under our experimental conditions (Fig. 5 D), but not its K⁺ permeability, as the lysosomal Δψ is positive in its value. Even at neutral pH, lysosomal Δψ is slightly positive (Fig. 5 D), suggesting that resting P_{Na} is slightly higher than resting P_{K}.

Notably, increasing cytoplasmic Ca²⁺ reduced lysosomal Δψ (Fig. 5 F and J; see also Fig. 5, A and B). Indeed, in small-sized vacuoles with only single LysoKVCa channel openings (Fig. S5 H), rapid and transient paxilline-sensitive decreases in lysosomal Δψ were observed (Fig. 5 G). Overexpression of SLO1 (Fig. S5, I–K) also resulted in reduced Δψ (Fig. 5, H and J). Furthermore, in endolysosomes expressing gain-of-function mutant SLO1 (SLO1 R207Q) channels (Montgomery and Meredith, 2012), which exhibited large basal currents at less positive voltages (Fig. S5, J–L), lysosomal Δψ was reversed to −20 to approximately −30 mV (Fig. 5, I and J). In SLO1- or SLO1 R207Q-expressing vacuoles, increasing cytoplasmic Ca²⁺ resulted in a much more negative lysosomal Δψ (−60 mV; Fig. 5, H–J). Collectively, these results suggest that LysoKVCa regulates lysosomal Δψ in response to changes in juxta-lysosomal Ca²⁺ levels.

**LysoKVCa** K⁺ homeostasis and cytosolic Ca²⁺ increase are both required for lysosomal Ca²⁺ store refilling

Lysosomal Δψ is a critical determinant of lysosomal ion homeostasis, including Ca²⁺ homeostasis, which is maintained at a luminal concentration of ∼0.5 mM (Morgan et al., 2011; Xu and Ren, 2015). To investigate the molecular mechanisms that regulate lysosomal Ca²⁺ stores, we recently developed a lysosomal Ca²⁺ refilling assay using ML-SA1, a membrane-permeable synthetic activator of lysosomal TRPML1 (or ML1) channels (Shen et al., 2012). In HEK293 cell lines stably expressing GCaMP3-ML1 (Fig. 6 A; HEK-GCaMP3-ML1 cells), bath application of ML-SA1 in a zero (<10 nM) Ca²⁺ external solution produced robust lysosomal Ca²⁺ release indicated by GCaMP3 fluorescence (Garrity et al., 2016). After 3–5 min of refilling time, secondary ML-SA1 responses were largely recovered (Fig. 6 B), suggesting that lysosomal Ca²⁺ stores were refilled (Garrity et al., 2016). Using this refilling assay, we recently reported that in contrast to previous findings, dissipating the H⁺ gradient does not block lysosomal Ca²⁺ refilling (Garrity et al., 2016). Instead, ER Ca²⁺ may refill lysosomal Ca²⁺ stores, via the presumed formation of ER–lysosome membrane contacts (Eden, 2016). Remarkably, dissipating the lysosomal K⁺ gradient with valinomycin resulted in a blockade of lysosomal Ca²⁺ store filling (Fig. 6, C and D; and Fig. S6 A), suggesting an essential role of lysosomal K⁺ gradient and efflux in refilling.

In theory, either a reduction in luminal Ca²⁺ or an increase in perilysosomal Ca²⁺ could serve as a trigger for Ca²⁺ refilling. Cytoplasmic Ca²⁺ increases have been hypothesized to regulate the formation and stabilization of ER–lysosome membrane contacts (Eden, 2016; Kilpatrick et al., 2017), potentially contributing to lysosomal Ca²⁺ refilling. To investigate the second possibility, we used a complementary refilling assay, based on the use of Oregon Green 488 Bapta-1-dextran (OG-BAPTA) dyes to directly measure lysosomal luminal Ca²⁺ contents (Garrity et al., 2016). Notably, chelating cytosolic Ca²⁺ completely blocked refilling (Fig. 6, E and F).

**LysoKVCa** is required for efficient lysosomal Ca²⁺ store refilling in normal physiology

We next tested the hypothesis that LysoKVCa mediates Ca²⁺-activated K⁺ permeability that participates in perilysosomal Ca²⁺-triggered lysosomal refilling. Remarkably, in the GCaMP3-based refilling assays, when we inhibited LysoKVCa acutely using membrane-permeable BK inhibitors (i.e., paxilline and quinidine) during refilling, lysosomal Ca²⁺ refilling was completely inhibited (Fig. 7, A–C). In contrast, refilling was not...
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• Affected by IBTX (a membrane-impermeable BK inhibitor) or NS1619 (a membrane-permeable BK opener; Fig. S6, D and E). Prolonged inhibition (3 h) of LysoKVCa abolished the naive Ca²⁺ release responses (Fig. S6, B, C, and E), suggesting that refilling is an ongoing process with constitutive Ca²⁺ release in the cells (Garrity et al., 2016). Note that paxilline did not directly...
affect the activity of lysosomal ML1 channels (Fig. S6, F and J) or luminal pH (Fig. S6, H and I).

Consistently, when LysoKVCa was genetically inactivated, as was seen in GCaMP7-ML1–transfected KCNMA1 KO MEFs, lysosomal Ca2+ refilling was also significantly reduced, but not abolished compared with WT MEFs (Fig. 7, D and G). Heterologous expression of WT SLO1-mCherry largely restored lysosomal refilling in KCNMA1 KO MEFs (Fig. 7, F and G). In contrast, when the Ca2+-sensitivity of SLO1-mCherry was abolished by mutations (SLO1M513I/D898A) in the Ca2+-binding sites (Bao et al., 2004; Fig. 7 E), the restoration effect was lost (Fig. 7, F and G). Hence, Ca2+ activation of LysoKVCa is specifically required for refilling of lysosomal Ca2+ stores.

To further investigate the role of LysoKVCa in regulating lysosomal Ca2+ store refilling, we studied refilling in cells loaded with Fura-2 using glycylphenylalanyl-2-naphthylamide (GPN), a lysosome-specific reagent that is widely used to deplete lysosomal Ca2+ stores (Berg et al., 1994), to induce ML1-independent Ca2+ release from lysosomes (Garritty et al., 2016). Blocking LysoKVCa in the refilling phase by using paxilline markedly attenuated the refilling response in both HEK293 cells and WT MEFs (Fig. 7, H–J; and Fig. S6, K–M) but had no effect on the residual refilling response observed in KCNMA1 KO MEFs (Fig. 7 J). It is of note that when MEK1/2 is active, the Ca2+ release response in the KCNMA1 KO cells or WT cells treated with paxilline acutely were comparable to those in non-treated WT cells (Fig. 7, D and H). Likewise, no difference was noted between WT and KCNMA1 KO cells in the OG-BAPT assay (Fig. 8). Hence, LysoKVCa is not required for ML1-mediated lysosomal Ca2+ release per se.

The recovery of lysosomal Ca2+ contents was profoundly inhibited by paxilline treatment in WT MEFs (Fig. 8, A and B). In contrast, the partial recovery seen in KCNMA1 KO MEFs was completely insensitive to paxilline (Fig. 8, A and B). Furthermore, overexpressing SLO1-mCherry, but not SLO1M513I/D898A-mCherry, in KCNMA1 KO MEFs largely restored the refilling of lysosomal Ca2+ contents (Fig. 8 C). Finally, pretreatment with paxilline dramatically decreased lysosomal Ca2+ contents in WT MEFs but had no effect on lysosomal Ca2+ stores or refilling in KCNMA1 KO MEFs (Fig. 8 D). Collectively, these results suggest that although there exist compensatory mechanisms for lysosomal store refilling in cells lacking KCNMA1, LysoKVCa is required for efficient refilling of lysosomal Ca2+ stores in normal physiology.

Genetic ablation or pharmacological inhibition of LysoKVCa leads to lysosomal dysfunction

Lysosomal Ca2+ and Δψ are important for lysosomal function (Medina et al., 2015; Wang et al., 2015; Xu and Ren, 2015). Lysosomal dysfunction is commonly associated with a compensatory increase in lysosome biogenesis, manifested as increased expression of essential lysosomal genes (Xu and Ren, 2015). For example, the expression of Lamp1, a housekeeping gene...
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for the lysosome, is elevated in most lysosomal storage diseases (Medina et al., 2015; Wang et al., 2015; Xu and Ren, 2015). Likewise, LysoTracker staining is also often elevated in LSD cells that yet have normal lysosomal pH (Xu and Ren, 2015). Indeed, both Lamp1 expression and LysoTracker staining were elevated significantly in KCN\(^{\text{MA1 K}}\)O MEFs relative to WT cells (Fig. 9, A and B), suggestive of an up-regulation of lysosomal biogenesis caused by lysosomal dysfunction. Note that lysosomal pH was normal in KCN\(^{\text{MA1 K}}\)O cells (Fig. S6 I).

Lysosomal Ca\(^{2+}\) signaling is required for the regulation of lysosomal proteolytic activity and cholesterol homeostasis, presumably via the regulation of lysosomal trafficking (Wang et al., 2015). Lysosomal proteolytic activity was measured using an assay that yields red fluorescence according to the proteolytic degradation of DQ-red-BSA, an artificial substrate (Yue et al., 2013). DQ-BSA degradation was found to be enhanced after complete starvation (withdrawal of both serum and amino acids in the culture medium) of Cos-1 cells (Fig. 9 C). Pharmacological inhibition of LysoK\(_{\text{VCa}}\) using membrane-permeable, but not membrane-impermeable, BK inhibitors resulted in a marked reduction in the starvation-induced enhancement of proteolytic activity (Fig. 9, C and D). Likewise, in KCN\(^{\text{MA1 K}}\)O MEFs, proteolytic activity was also attenuated (Fig. 9 E), and the remaining activity became resistant to BK inhibition (Fig. 9 E). Lysosomal cholesterol metabolism is regulated by lysosomal Ca\(^{2+}\), and cholesterol accumulation is observed in multiple LSD cells, including ML-IV and NPC cells (Wang et al., 2015; Xu and Ren, 2015). Compared with WT cells, KCN\(^{\text{MA1 K}}\)O MEFs exhibited mild but significant cholesterol accumulation (Fig. 9, F and G), suggesting that lysosomal BK is required for efficient cholesterol transport in the lysosomes. Collectively, these results suggest that LysoK\(_{\text{VCa}}\) is required for the normal function of lysosomes.

Figure 7. **LysoK\(_{\text{VCa}}\) and its Ca\(^{2+}\) sensitivity regulate the refilling of lysosomal Ca\(^{2+}\) stores.** (A and B) Acute application of paxilline (A) and quinidine (B) abolished the second ML-SA1-induced responses. Prolonged washout for 10–15 min led to a partial recovery of the responses. (C) Lysosome Ca\(^{2+}\) refilling in HEK-GCaMP3-ML1 cells treated with paxilline, quinidine, and IBTX. (D) Compared with WT MEFs, ML-SA1–stimulated refilled responses were reduced in GCaMP7-ML1-expressing KCNMA1 KO MEFs. (E) Lyso-SLO1\(^{\text{M513I/D898A}}\) currents at different concentrations of Ca\(^{2+}\) (0.1 and 10 µM). (F and G) Lysosomal refilling in GCaMP7-ML1–expressing KCNMA1 KO MEFs that were transfected with WT SLO-mCherry and SLO1\(^{\text{M513I/D898A-mCherry}}\). (H) GPN-induced refilled (the second) Ca\(^{2+}\) response, measured with Fura-2 imaging, was reduced in KCNMA1 KO MEFs. (I) Paxilline effects in lysosomal refilling in WT MEF cells. (J) Mean refilling responses in WT and KCNMA1 KO MEF cells. Statistical comparisons were made with variance analysis (Student’s t test). **, P < 0.01; ***, P < 0.001. Error bars indicate SEM.
BK channels are negative-feedback regulators of \( \text{Ca}^{2+} \) overload and membrane hyperexcitability in excitable cells. We demonstrated in the current study that BK channels are functionally present in the lysosomes of both excitable and nonexcitable cells. In contrast, the expression of BK channels at the plasma membrane and early endosomes is absent or very low in the nonexcitable cells. Therefore, BK channels are specifically targeted to the lysosomes, and endogenous SLO1 proteins are bona fide lysosomal channels in many cell types, including MEFs. In another contrast, other plasma membrane background \( K^+ \) conductances are undetectable in the lysosomes. Using our lysosome-specific \( \text{Ca}^{2+} \) release and content measurement assays, we showed that lysosomal BK channels are required for efficient refilling of lysosomal \( \text{Ca}^{2+} \) stores and normal function of lysosomes.

An unexpected finding in the current study is that in the isolated enlarged lysosomes that are used for our electrophysiological measurements, lysosomal \( \Delta \psi \) is positive. Our measurement was based on a high \([\text{Na}^+]_l/\text{K}^+]_i\) ratio previously determined from ionic composition analysis of isolated lysosomes (Wang et al., 2012). If the \([\text{Na}^+]_l/\text{K}^+]_i\) ratio were lower in intact cells (Steinberg et al., 2010), lysosomal \( \Delta \psi \) would be still positive. Several previous studies have reported that lysosomal \( \Delta \psi \) are negative, with the values scattered between \(-10\) and \(-100\) mV in different studies and cell types (Morgan et al., 2011). For example, using fluorescence resonance energy transfer–based indicators, one recent study reported that phagolysosomal \( \Delta \psi \) is \(-19\) mV in intact single cells (Koivusalo et al., 2011). Recent studies suggest that lysosomal pH is highly heterogeneous, with some of the primary lysosomes completely unacidified (Bright et al., 2016; Johnson et al., 2016). Therefore, our measured lysosomal \( \Delta \psi \) based on fixed luminal composition might not be faithfully extrapolated in vivo. It is possible that certain physiological regulators of lysosomal \( \Delta \psi \) are lost in our isolated lysosome recordings. However, based on our current study, such unidentified physiological regulators would have to up-regulate lysosomal \( K^+ \) permeability, as both \( P_{\text{Na}} \) and \( P_{\text{K}} \) contribute to positive \( \Delta \psi \).

The vacuolin-1–enlarged vacuoles that we used for patch-clamping most likely originated primarily from late endosomes and lysosomes, as they were positive for Lamp1, but not for ER or mitochondrial markers (Fig. S1 A). Admittedly, the membrane properties of enlarged vacuoles may not be identical to native lysosomal membranes. \( K_{\text{VCA}} \) currents are undetectable in the early endosomes, and the background and resting \( K^+ \) conductances at the plasma membrane are also undetectable in the lysosome. Therefore, enlarged endolysosomes do not accumulate any nonlysosomal channel proteins in their limited/perimeter membranes, and the whole-endolysosome technique is reasonably specific in detecting ion channels on the lysosomal membranes. Using this technique, we found that LysoKVCa represents the major \( K^+ \) conductance in the lysosome, although we estimated based on the single-channel recording data that there are few channels per lysosome (approximately one channel per \( 1 \mu \text{m}^2 \) of lysosomal membrane). However, because of the large single-channel conductance, opening of single LysoKVCa currents was sufficient to confer significant changes in lysosomal \( \Delta \psi \). Unlike the plasma membrane, in which the opening of ion channels would not significantly change the concentration gradients of ions, opening of LysoKVCa for 100 ms is sufficient to cause the dissipation of the lysosomal \( K^+ \) gradients. Hence the mean open and close times of single LysoKVCa channels may have large impacts on lysosomal physiology.

It is likely that the primary function of LysoKVCa, in lysosomes, is to regulate lysosomal \( \Delta \psi \), which in turn regulates lysosomal \( \text{Ca}^{2+} \) refilling and signaling (Fig. 10). In the absence of other major \( K^+ \) conductances at rest, even with submaximal activation, a brief opening of a single large-conductance \( K^+ \) channel in the small-sized lysosome can change \( \Delta \psi \) rapidly and effectively. During the course of this study, it was reported that

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**Discussion**

BK channels are negative-feedback regulators of \( \text{Ca}^{2+} \) overload and membrane hyperexcitability in excitable cells. We demonstrated in the current study that BK channels are functionally present in the lysosomes of both excitable and nonexcitable cells. In contrast, the expression of BK channels at the plasma membrane and early endosomes is absent or very low in the nonexcitable cells. Therefore, BK channels are specifically targeted to the lysosomes, and endogenous SLO1 proteins are bona fide lysosomal channels in many cell types, including MEFs. In another contrast, other plasma membrane background \( K^+ \) conductances are undetectable in the lysosomes. Using our lysosome-specific \( \text{Ca}^{2+} \) release and content measurement assays, we showed that lysosomal BK channels are required for efficient refilling of lysosomal \( \text{Ca}^{2+} \) stores and normal function of lysosomes.

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TMEM175 proteins may mediate a background K⁺ leak conductance. However, this background K⁺ current was very small in the original study (20 pA at 100 mV; Cang et al., 2015) and barely detectable in our current experimental conditions. Future studies may reveal the relative contributions of LysoKVCa, TMEM175, and other lysosomal K⁺ conductances to lysosomal Δψ in various cell types under various cellular conditions. It is important to note that a positive lysosomal Δψ favors the channel openings of LysoKVCa in response to small increases in juxta-lysosomal Ca²⁺ that occur during membrane fusion and fission events. Reversal of Δψ may increase the driving force for MLI-mediated Ca²⁺ release initially, but then deactivate LysoKVCa to shape the duration of lysosomal Ca²⁺ signaling. However, the role of LysoKVCa in lysosomal Ca²⁺ refilling is independent of its effect on lysosomal Ca²⁺ release.

The mechanisms by which the 5,000-fold Ca²⁺ gradient across lysosomal membranes is established and maintained are not clear (Xu and Ren, 2015). We recently reported that the ER serves as a primary source of lysosomal Ca²⁺, presumably via ER–lysosome membrane contacts (Garrity et al., 2016). However, it remains to be determined how the Ca²⁺ refilling process is triggered. Theoretically, either a reduction in luminal [Ca²⁺] or an increase of juxta-lysosomal [Ca²⁺] could trigger the refilling process. In the case of ER Ca²⁺ refilling, STIM proteins mediate luminal Ca²⁺ sensing, and activation of STIM1 triggers the formation of ER–plasma membrane junction and subsequent ORAI channel openings. For lysosomal Ca²⁺ refilling, given that refilling is completely blocked by the chelation of cytoplasmic Ca²⁺, the latter is more likely the case. However, it is not clear whether Ca²⁺ chelation may affect the formation of ER–lysosome membrane contact sites or the local Ca²⁺ concentrations at the contact sites. Nevertheless, we provide evidence that LysoKVCa could serve as an effector for juxta-lysosomal Ca²⁺, mediating store refilling. Presumed local Ca²⁺ increases in the ER–lysosome membrane contact sites during the seemingly quiescent refilling phase, but not increases in bulk cytoplasmic Ca²⁺ (e.g., during SERCA inhibition), are required for refilling (Garrity et al., 2016), in which presumed LysoKVCa activation may play a role. However, the mechanisms by which LysoKVCa and Δψ regulate lysosomal Ca²⁺ refilling are unknown, largely because of the lack of the knowledge on the molecular identity of the Ca²⁺ uptake transporters and the regulatory mechanisms of ER–lysosome interaction. Based on our results in the current study, recent studies on ER–lysosome interaction (Phillips and Voeltz, 2016), and previous Ca²⁺ uptake studies on isolated lysosomes (Lemons and Thoene, 1991), we hypothesize that ER

Figure 9. LysoKVCa is required for lysosome function. (A) Western blot analysis of Lamp1 expression in WT and KCNMA1 KO MEFs, (B) LysoTracker staining in WT and KCNMA1 KO MEFs. Bar, 10 µm. (C) Confocal imaging of DQred-BSA in starved Cos-1 cells (amino acid + serum starvation) in the presence of paxilline (10 µM), quinidine (500 µM), or IBTX (100 nM). Bar, 10 µm. (D) Normalized proteolytic index for starved WT Cos-1 cells treated with paxilline or quinidine. (E) Normalized proteolytic index for starved WT and KCNMA1 KO MEFs treated with paxilline. (F) Cholesterol levels, detected with filipin staining, in WT, KCNMA1 KO, and NPC1 KO MEFs. Bar, 50 µm. (G) Normalized filipin density in WT, KCNMA1 KO, and NPC1 KO MEFs. Means ± SEM are shown in A, D, E, and G. *, P < 0.05; **, P < 0.01.
refilling of lysosomal stores is a regulated two-step process. First, lysosome store depletion may trigger an arrangement of ER–lysosome contact configuration (Phillips and Voeltz, 2016). Perilysosomal Ca\(^{2+}\) increases were proposed to regulate ER–lysosome membrane contact, but the direct evidence is still lacking (Eden, 2016). Second, at these relatively stable, functional ER–lysosome contact sites, a passive Ca\(^{2+}\) transport process can occur from ER to lysosomes, because of the large chemical gradient of Ca\(^{2+}\) that is created when lysosome stores are actively depleted. It is conceivable that both steps are dependent on lysosomal \(\Delta \psi\), either directly or indirectly. For example, it is recently reported that \(\Delta \psi\) may affect dynamics of phosphoinositide (Zhou et al., 2015), which is known to regulate the interaction of lysosomes with other organelles (Chu et al., 2015). Furthermore, we show that LysoKVCa is required for the lysosomal export of cholesterol, which is known to affect ER–lysosome interaction and the activities of lysosomal channels and transporters (Xu and Ren, 2015). Future studies may reveal whether lysosomal \(\Delta \psi\) regulates ER–lysosome interaction or Ca\(^{2+}\) uptake mechanisms via cholesterol export and signaling. Testing these hypotheses comprehensively will require expanding our knowledge of transporters and channels in endolysosomal membranes and the development of accurate methods of measuring endolysosomal potentials in intact cells.

### Materials and methods

#### Molecular biology

Mouse SLO1\(^{R207Q-YFP}\), SLO1\(^{L1233I/L1234A}\), SLO1\(^{K488M/L489M/L733V/L734V}\), and SLO1\(^{M513I/D898A}\) were generated from mouse SLO1-YFP (a gift from R. Brenner, University of Texas, San Antonio, TX) with the Quick-Change Lightning Site-Direct Mutagenesis kit (Agilent Technologies). GFP-tagged \(\beta_1, \beta_2,\) and \(\beta_4\) constructs were provided by T. Hoshi (University of Pennsylvania, Philadelphia, PA). Genetically encoded Ca\(^{2+}\) indicator GCaMP3 and GCaMP7 was fused directly to the N terminus of ML1 (GCaMP3-ML1 and GCaMP7-ML1), as described previously (Shen et al., 2012). All constructs were confirmed by sequencing, and protein expression was verified by Western blotting and fluorescence imaging.

#### Mouse lines

KCNMA1 KO (Kcnma\(^{−/−}\) or Slo1\(^{−/−}\)) mice were generated and characterized as described previously (Montgomery and Meredith, 2012). All animal experiments were conducted using an approved protocol (#6577) and Institutional Animal Care Guidelines of the University of Michigan.

#### Mammalian cell culture

Cos-1 cells, HEK-293T cells, CV1 cells, A7r5 cells, and BECs were grown at 37°C in a 1:1 mixture of DMEM supplemented with 10% FBS (Gibco) in a humidified 5% CO\(_2\) incubator. INS-1 cells were cultured in RPMI-1640 (11 mM glucose) supplemented with 10% FBS and 50 mM \(\beta\)-mercaptoethanol. HEK293 cells stably expressing GCaMP3-ML1 (HEK-GCaMP3-ML1 cells) were generated using the Flip-In T-Rex 293 cell line (Invitrogen). HEK-GCaMP3-ML1 cells were grown in Tet-free FBS, and GCaMP3-ML1 expression was induced using doxycycline.

#### MEF isolation

Sterilized skin specimens from WT and KCNMA1 KO mice were minced into small fragments (\(\sim 1\) mm\(^2\)) and incubated in 0.05% trypsin and 0.2% collagenase at 37°C for 1 h. Single fibroblasts, isolated by repeated pipetting, were maintained in DMEM supplemented with 20% FBS and an antibiotic and antifungal cocktail (Thermo Fisher Scientific) at 37°C in a 5% CO\(_2\) incubator.

#### Primary cortical neurons

Mouse pups were killed within 2 d of birth, and brains were dissected in ice-cold HBSS. After the removal of meninges, the dissected brains were minced into small fragments (\(\sim 1\) mm\(^3\)) and incubated in 0.05% trypsin and 0.2% collagenase at 37°C for 1 h. Single fibroblasts, isolated by repeated pipetting, were maintained in DMEM supplemented with 20% FBS and an antibiotic and antifungal cocktail (Thermo Fisher Scientific) at 37°C in a 5% CO\(_2\) incubator.
mg/ml fungizone) at 37°C in 5% CO₂. Whole-endolysosomal recordings were performed in neurons that were cultured for more than 10 d.

Lysosome isolation by subcellular fractionation

Lysosomes were isolated as described previously (Wang et al., 2012). In brief, cells lysates were obtained by Dounce homogenization in a homogenizing buffer (HM buffer: 0.25 M sucrose, 1 mM EDTA, and 10 mM Hepes, pH 7.0). Nuclei and intact cells were removed by centrifugation at 1,900 g at 4°C for 10 min. Supernatant was then collected and further centrifuged through a Percoll density gradient using a Beckman L-70 centrifuge and a 7.01 Ti rotor. The Percoll gradient was prepared with 0.7 ml of 25 M sucrose at the bottom, 6 ml of 18% Percoll (mixture of 1.08 ml Percoll and 4.92 ml HM buffer) in the middle, and 1 ml postnuclear supernatants on top. The centrifuge was performed at 4°C for 1 h at 67,200 g (14,000 rpm). Heavy membrane fractions containing lysosomes were concentrated at the bottom of the Percoll gradient. They were carefully collected and laid over a discontinuous iodixanol gradient. The centrifuge tube was loaded with 0.5 ml of 2.5 M sucrose at the bottom, and the iodixanol gradient was generated through dilution of iodixanol (by HM buffer) to a final vol/vol (from bottom of the centrifuge tube to top) of 27, 22.5, 19, 16, 12, and 8%. The heavy membrane fraction (1 ml) was laid carefully on top, and the fractionation was performed at 130,000 g (44,200 rpm) for 2.5 h at 4°C. After fractionation, the sample was carefully divided into fractions of 0.5 ml. Fractions containing the highest purity of lysosomes were determined using Western blotting and used for subsequent analysis.

Western blotting

Standard Western blotting procedures were used. In brief, cells were lysed with ice-cold RIPA buffer (Boston BioProducts) in the presence of 1x protease inhibitor cocktail (Sigma-Aldrich), 1 mM NaF, and 1 mM Na₃VO₄. Total cell lysates were mixed with 2x SDS loading buffer and boiled at 95°C for 10 min. Protein samples (10–100 µg) were then loaded and separated on 4–12% gradient SDS-PAGE gels (Invitrogen) and transferred to PVDF membranes. The membranes were blocked for 1 h with 1% BSA in PBS supplemented with 0.1% Tween20 and incubated with antibodies against GFP (1:10,000; Covance), Lamp1 (1:1,000; Developmental Studies Hybridoma Bank), EEA1 (1:1,000; Abcam), and Complex II (1:1,000; Developmental Studies Hybridoma Bank), EEA1 (1:1,000; Abcam). (1:1,000; Developmental Studies Hybridoma Bank), EEA1 (1:1,000; Abcam). Bound antibodies were detected using HRP-conjugated anti-rabbit (65-6120) or anti-mouse (62-6520) secondary antibodies (1:5,000; Invitrogen) and enhanced chemiluminescence reagent (GE Healthcare). Band intensities were quantified with ImageJ software.

Immunofluorescent labeling and confocal imaging

Cells were grown on glass coverslips, fixed with 4% PFA, and permeabilized with 0.3% Triton X-100. They were then blocked with 1% BSA in PBS. After three washes with PBS, the slides were processed for imaging using a confocal microscope (TCS SP5: Leica Biosystems) with a 100× oil objective NA 1.40 (HCX PL APO: Leica Biosystems). PMT detector was used for DAPI signal (excitation, 405 nm; emission, 410–440 nm); Hyd detector was used for GFP (excitation, 488 nm; emission, 492–535 nm) and mCherry/Alexa Fluor 568 (excitation, 561 nm; emission, 575–625 nm) signals. Images were acquired with LAS AF software (Leica Biosystems) and analyzed with ImageJ and Photoshop CS6 (Adobe).

GCaMP3-Ca²⁺ imaging

Tet-On HEK-GCaMP3-ML1 stable cells were used for Ca²⁺ imaging. Doxycycline (0.01 µg/ml) was applied 20–24 h before the experiments to induce GCaMP3-ML1 expression. Cells were trypsinized and plated on coverslips 4–6 h before experiments. GCaMP3 fluorescence intensity at 470 nm (F₄70) was monitored and recorded at RT (21–23°C) using a EasyRatio Pro system (Photon Technology International), which includes an invert microscope (Olympus 1X71) with a 20x/0.75 objective lens (Olympus UApo/340), a high-resolution CoolSNAP HQ² CCD camera (Photometrics), and a high-speed DeltaRam X monochromator (Photon Technology International) operated with EasyRatioPro software 1.12.121.86 (Photon Technology International). During recording, cells were bathed in Tyrode’s solution containing 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 20 mM Hepes, pH 7.4. Lysosomal Ca²⁺ release was measured by briefly switching to a 0 Ca²⁺ solution, which contained 145 mM NaCl, 5 mM KCl, 3 mM MgCl₂, 10 mM glucose, 1 mM EGTA, and 20 mM Hepes, pH 7.4; free Ca²⁺ concentration is estimated to be <10 nM based on MaxChelator software (Shen et al., 2012). Alterations of Ca²⁺ levels were normalized and shown as ΔF/F₀, where F₀ is baseline F, and ΔF is changes in F upon treatment.

OG-BAPTA imaging

Cells were loaded with OG-BAPTA (100 µg/ml; Thermo Fisher Scientific) at 37°C in the culture medium for 4–12 h, and then pulsed/chased for additional 4–16 h. Fluorescence imaging was performed at 37°C with a Spinning Disc Confocal Imaging System, which includes an IX81 inverted microscope (Olympus), a 60x objective NA 1.42 (Olympus; PlanApo N), a CSU-X1 scanner (Yokogawa Electric Corporation), and an iXon EM-CCD camera (Andor). Images were acquired and analyzed with MetaMorph Advanced Imaging acquisition software v. 7.7.8.0 (Molecular Devices). In vitro calcium-binding (Kᵢ) affinities of OG-BAPTA were determined using KCl-based solutions (140 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, 10 mM MES, and 0 or 1 mM BAPA) adjusted to different pH levels (4.5, 5.0, 6.0, and 7.0). By varying the amount of added Ca²⁺ (x = 0–10 mM), solutions with different pH and free [Ca²⁺] were made based on MaxChelator software. OG-BAPTA (5 µg/ml) fluorescence for each solution was obtained to plot the calibration curve (Christensen et al., 2002; Dickson et al., 2012; Morgan et al., 2015). In cells that were pretreated with ionomycin, nigericin, and valinomycin, in vivo minimal and maximal fluorescence (Fₘₐₚ and Fₘₜₐₚ) were determined by perfusing the cells with 0 or 10 mM Ca²⁺ external solutions, respectively (Christensen et al., 2002; Dickson et al., 2012; Morgan et al., 2015). Lysosomal [Ca²⁺] were at different pH were determined using the following calibration equation: [Ca²⁺] = Kᵢ × (F – Fₘₚ)/(Fₘₜₐₚ – F).

Lysosomal pH measurement

Cells seeded on coverslips were pulsed with OG-BAPTA (100 µg/ml) at 37°C in the culture medium for 6 h and chased for additional 12 h (Johnson et al., 2016). Cells were then bathed in Tyrode’s solution. Images were captured at RT using an EasyRatio Pro system (Photon Technology International). Fluorescence was continuously recorded at RT with alternate excitation wavelengths of 480 and 430 nm (F₄₈₀ and F₄₃₀, respectively). To convert fluorescence ratios (F₄₈₀/F₄₃₀) to pH values, a pH standard curve was constructed using isotonic K⁺ solutions (145 mM KCl, 5 mM glucose, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM Hepes, and 10 mM MES, adjusted to various pH values ranging from 4.0 to 8.0) containing 10 µM nigericin and 10 µM monensin (Johnson et al., 2016).

Whole-endolysosome electrophysiology

Endolysosomal electrophysiology was performed on isolated endolysosomes using a modified patch-clamp method (Dong et al., 2008). In brief, cells were treated with 1 µM vacuolin-1, a lipid-soluble polycyclic triazine that can increase the size of endosomes and lysosomes selectively (Cerny et al., 2004), for at least 1 h and up to 12 h. Large vacuoles...
were conducted at RT (21–23°C), and all recordings were analyzed in currents were digitized at 10 kHz and filtered at 2 kHz. All experiments with pClamp 10.0 software (Axon Instruments). Whole-endolysosome recordings were then performed on enlarged vacuoles from cells that were released into the dish. Note that the membrane properties of enlarged vacuoles may not be identical to native lysosomal membranes. However, the whole-endolysosome technique is thus far the only feasible approach for directly studying lysosomal channels (Xu and Ren, 2015).

Unless otherwise stated, the bath (internal/cytoplasmic) solution contained 140 mM K+-gluconate, 4 mM NaCl, 2 mM MgCl2, and 10 mM Hepes (pH adjusted with KOH to 7.2). Solutions with 0.1–10 µM free Ca2+ were prepared by combining various concentrations of EGTA and CaCl2, as calculated with Maxchelator. Bath solutions with 100–1,000 µM Ca2+ contained various amounts of CaCl2 without EGTA. pH adjustments (6.0, 7.2, and 9.0) were made in either 0.1 µM MES, and 10 mM glucose (pH adjusted to 4.6 with NaOH). To avoid contamination of background Cl− currents existing in some patches, low-Cl− pipette solutions were used in many experiments, in which Na-glucuronate was used as a substitute for NaCl. All bath solutions were applied via a fast perfusion system to achieve a complete solution exchange within a few seconds. Data were acquired with an Axopatch 2A patch-clamp amplifier and a Digidata 1440 digitizer and recorded with pClamp software. Whole-endolysosome currents were digitized at 10 kHz and filtered at 2 kHz. All experiments were conducted at RT (21–23°C), and all recordings were analyzed in pCLAMP10 (Axon Instruments) and Origin 8.0 (OriginLab) software.

Endolysosomal excised-patch electrophysiology
For giant excised-patch, endolysosomal luminal-side-out recordings, polished pipette electrodes (resistance, 1–2 MΩ) were filled with a solution containing 140 mM K+-gluconate, 4 mM NaCl, 2 mM MgCl2, 1 mM CaCl2, and 10 mM Hepes (pH adjusted with KOH to 7.2). The bath solution contained 153 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Hepes, 10 mM MES, and 10 mM glucose (pH adjusted to 4.6 with NaOH). The pipette (luminal) solution contained 145 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Hepes, 10 mM MES, and 10 mM glucose (pH adjusted with KOH to 7.2). The bath solution contained 153 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 20 mM Hepes, and 10 mM glucose, pH 7.4. Excised-patch, endolysosomal cytoplasmic-side-out (Dong et al., 2008) recordings were performed in isolated enlarged endolysosomes with the same pipette and bath solutions that were used in the whole-endolysosome recordings.

Whole-cell electrophysiology
Whole-cell recordings were performed in Cos-1 cells via pipette electrodes (resistance 3–5 MΩ) filled with the following solution: 140 mM K+-gluconate, 4 mM NaCl, 2 mM MgCl2, 0.1 mM CaCl2 (unless otherwise indicated), and 10 mM Hepes (pH adjusted with KOH to 7.2). The standard extracellular bath solution (modified Tyrode’s solution) contained 153 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM Hepes, 10 mM MES, and 10 mM glucose (pH adjusted to 4.6 with NaOH). Data were acquired with an Axopatch 2A patch-clamp amplifier and a Digidata 1440 digitizer and recorded with pClamp software.

DQ-BSA proteolytic assay
DQ-red-BSA was used as an artificial substrate to evaluate lysosomal proteolytic degradation (Yue et al., 2013). In brief, cells were treated with DQ-red-BSA (10 µg/ml; Thermo Fisher Scientific) for 2 h at 37°C in complete culture medium. After removal of extracellular DQ-red-BSA by washing twice with PBS, cells were starved in amino acid– and serum-free DF12 medium (US Biological) for 2 h to trigger autophagic degradation. To examine the effects of BK blockers on lysosomal proteolytic activities, paxilline (10 µM), quinidine (500 µM), or IBTX (100 nM) was applied during starvation. Cells were then fixed with 4% PFA for 15 min at RT, washed twice with PBS, and mounted on slides with Fluoromount-G (SouthernBiotech). DQ-red-BSA fluorescence was detected with a 561/607 filter set using the Spinning Disc Confocal Imaging System under an 60x oil objective NA 1.42 (Olympus; PlanApo N). Images were acquired and analyzed with MetaMorph Advanced Imaging acquisition software v. 7.7.8.0 (Molecular Devices). Quantification was performed using ImageJ.

Filipin staining
Cells were fixed in 4% PFA for 1 h, washed three times with PBS, and then incubated with 1.5 mg/ml glycine in PBS for 10 min to quench the PFA. Cells were then stained for 2 h with 0.05 mg/ml filipin in PBS supplemented with 10% FBS. All procedures were conducted at RT (21–23°C). Images were obtained using a fluorescence microscope with a UV filter. Filipin intensity was calculated using ImageJ.

Reagents
All reagents were dissolved and stored in DMSO or water. DQ-BSA-red was from Life Technologies; anti-SLO-1 antibodies were purchased from NeuroMab; vacuolin-1 was from Calbiochem; ML-SA1 was from Princeton BioMolecular Research Inc.; quinidine, paxilline, chlorotoluyl tosylate, NS619, IBTX, TEA chloride, and filipin complex were purchased from Sigma-Aldrich.

Data analysis
Data are presented as mean ± SEM. Statistical comparisons were performed using Student’s t test and analysis of variance (ANOVA). P-values <0.05 were considered statistically significant.

Online supplemental material
Fig. S1 shows that vacuolin-1 selectively enlarges endosomes and lysosomes. Fig. S2 shows that LysoKVCa currents are present in a variety of cell types. Fig. S3 shows the regulation of LysoKVCa by Ca2+, Mg2+, and membrane voltages. Fig. S4 shows the regulation of LysoKVCa by BK modulators and pH. Fig. S5 shows cell type–specific properties of LysoKVCa conferred by lysosomal localization of auxiliary β subunits. Fig. S6 shows the regulation of lysosomal Ca2+ refilling by LysoKVCa.

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Author contributions: W. Wang conceived, designed, and performed the electrophysiology and Ca2+ imaging experiments; W. Wang and H. Xu drafted the manuscript with inputs from all authors; X. Zhang and M. Gu performed electrophysiological experiments; Q. Gao performed the proteolytic assay; M. Lawas performed the Ca2+ imaging experiments; Q. Gao, L. Yu, and X. Cheng performed the confocal imaging experiments; N. Sahoo and X. Li performed the biochemical experiments; S. Ireland and A. Meredith provided reagents; H. Xu supervised the project.
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