Lysosomes shape Ins(1,4,5)P3-evoked Ca2+ signals by selectively sequestering Ca2+ released from the endoplasmic reticulum

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Summary

Most intracellular Ca2+ signals result from opening of Ca2+ channels in the plasma membrane or endoplasmic reticulum (ER), and they are reversed by active transport across these membranes or by shuttling Ca2+ into mitochondria. Ca2+ channels in lysosomes contribute to endo-lysosomal trafficking and Ca2+ signalling, but the role of lysosomal Ca2+ uptake in Ca2+ signallung is unexplored. Inhibition of lysosomal Ca2+ uptake by dissipating the H+ gradient (using bafilomycin A1), perforating lysosomal membranes (using glycyl-L-phenylalanine 2-naphthylamide) or lysosome fusion (using vacuolin) increased the Ca2+ signals evoked by receptors that stimulate inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] formation. Bafilomycin A1 amplified the Ca2+ signals evoked by photolysis of caged Ins(1,4,5)P3 or by inhibition of ER Ca2+-pumps, and it slowed recovery from them. Ca2+ signals evoked by store-operated Ca2+ entry were unaffected by bafilomycin A1. Video-imaging with total internal reflection fluorescence microscopy revealed that lysosomes were motile and remained intimately associated with the ER. Close association of lysosomes with the ER allows them selectively to accumulate Ca2+ released by Ins(1,4,5)P3 receptors.

Key words: Ca2+, Endoplasmic reticulum, Ins(1,4,5)P3 receptor, Lysosome, Store-operated Ca2+ entry

Introduction

Because Ca2+ can be rapidly moved across biological membranes to generate spatially organized increases in cytosolic free Ca2+ concentration ([Ca2+]i) (Smith and Parker, 2009), it is a versatile and ubiquitous intracellular messenger (Berridge et al., 2003; Rizzuto and Pozzan, 2006). Most Ca2+ signals are generated by regulated opening of Ca2+-permeable channels within either the plasma membrane (PM) or the membranes of intracellular organelles, most commonly the endoplasmic reticulum (ER). Within the latter, inositol 1,4,5-trisphosphate receptors [Ins(1,4,5)P3R] are almost invariably expressed (Foskett et al., 2007). In addition, the ER often expresses ryanodine receptors (RyR) and a variety of other Ca2+-permeable channels (Taylor and Dale, 2012). Regulated opening of these channels allows Ca2+ to flow rapidly into the cytosol. This, together with the high concentration of cytosolic Ca2+ buffers, allows an open Ca2+ channel to generate a local, but substantial, increase in [Ca2+]i, that decays within about a hundred nanometres of the channel and persists only for as long as the channel is open (Shuai and Parker, 2005). These local microdomains of Ca2+ around the mouths of open channels are the fundamental units of Ca2+ signalling. They allow different channels to direct Ca2+ to different effector systems (Rizzuto and Pozzan, 2006). They allow Ca2+-mediated communication between Ca2+ channels to facilitate regenerative growth of Ca2+ signals by Ca2+-induced Ca2+ release, usually via Ins(1,4,5)P3R (Calcraft et al., 2009; Rahman et al., 2009; Smith and Parker, 2009) or RyR (Bailou et al., 2010; Cheng and Lederer, 2008). And where Ca2+ channels are close to another membrane, they allow Ca2+ to be selectively directed to proteins that remove Ca2+ from the cytosol. The Ca2+-pump of the ER, for example, can sequester Ca2+ entering via store-operated Ca2+ entry (SOCE) (Parekh and Putney, 2005) and mitochondria can selectively accumulate Ca2+ released by Ins(1,4,5)P3R (Csordás et al., 2010) or entering via SOCE (Parekh and Putney, 2005). These intimate relationships between Ca2+ channels and proteins that sequester, release or respond to Ca2+ endow the fundamental units of Ca2+ signalling, the local increase in [Ca2+]i, evoked by opening of a single channel, with the versatility needed to regulate almost every cellular activity (Berridge et al., 2003).

The membranes that have attracted most attention in the context of Ca2+ signalling are the PM, the ER and the inner mitochondrial membrane. All three membranes express proteins that remove Ca2+ from the cytosol and so terminate Ca2+ signals. Mitochondrial Ca2+ uptake additionally links cytosolic Ca2+ signals to oxidative phosphorylation (Jouaville et al., 1999), mitochondrial motility (Yi et al., 2004) and cell death (Szalai et al., 1999). The ER and PM express a wide variety of Ca2+ channels that can generate cytosolic Ca2+ signals (Taylor and Dale, 2012; Taylor et al., 2009). The recent identification of regulated Ca2+ channels in lysosomes, notably the two-pore channels (TPC) gated by nicotinic acid dinucleotide phosphate (NAADP) (Calcraft et al., 2009) and various transient receptor potential (TRP) channels (Dong et al., 2010), has suggested that they too may contribute to cytosolic Ca2+ signalling. Lysosomes are the main degradative compartment of the cell, they contain...
substantial amounts of Ca²⁺ (Christensen et al., 2002; Morgan et al., 2011), membrane trafficking within the endo-lysosomal pathway is regulated by Ca²⁺ (Luzio et al., 2010), and dysfunctional lysosomes cause devastating lysosomal storage diseases (Vellodi, 2005), some of which are associated with defective lysosomal Ca²⁺ regulation (Lloyd-Evans and Platt, 2011; Morgan et al., 2011; Pereira et al., 2010). There is, therefore, a growing recognition that lysosomal Ca²⁺ channels contribute to the genesis of cytosolic Ca²⁺ signals and to regulation of endo-lysosomal membrane trafficking. However, the mechanism of Ca²⁺ uptake by mammalian lysosomes is not resolved (Lloyd-Evans and Platt, 2011) and nor has the contribution of lysosomal Ca²⁺ uptake to the shaping of Ca²⁺ signals evoked by conventional Ca²⁺ signalling pathways been established. Here, we show that intimate and dynamic associations between lysosomes and the ER allow lysosomes selectively to accumulate Ca²⁺ released from the ER. Lysosomes thereby shape the cytosolic Ca²⁺ signals evoked by activation of Ins(1,4,5)P₃R without affecting those evoked by SOCE.

**Results**

**Dissipating the lysosomal H⁺ gradient enhances carbachol-evoked Ca²⁺ signals**

Activation of endogenous muscarinic receptors in human embryonic kidney (HEK) cells with carbachol (CCh) stimulates phospholipase C (PLC), formation of Ins(1,4,5)P₃, release of Ca²⁺ from intracellular stores, and stimulation of Ca²⁺ entry (Tovey et al., 2008). The initial release of Ca²⁺ from intracellular stores is entirely mediated by Ins(1,4,5)P₃R and unaffected by inhibition of RyR or TPC (supplementary material Fig. S1).

Ca²⁺ accumulation by acidic organelles, including lysosomes, requires a transmembrane H⁺ gradient. It is probably mediated by a Ca²⁺/H⁺ exchanger or indirectly via a Ca²⁺/Na⁺ exchanger. The identities of these exchangers in mammalian cells are unknown (Klemper, 1985; Morgan et al., 2011). We used bafilomycin A₁ to inhibit the vacuolar type H⁺-ATPase (V-ATPase) (Yoshimori et al., 1991), dissipate the H⁺ gradient and so prevent Ca²⁺ accumulation by acidic organelles (Fig. 1A). LysoTracker Red, a weak base that accumulates in acidic organelles (Bucci et al., 2000), substantially colocalised with a GFP-tagged lysosomal membrane protein (lysosomal-associated membrane protein 1; LAMP1–GFP) (Fukuda, 1991) transiently expressed in HEK cells (supplementary material Fig. S2). Bafilomycin A₁ (1 μM, 1 h) almost abolished staining with LysoTracker Red without affecting the distribution of LAMP1–mCherry (supplementary material Fig. S3). This demonstrates that lysosomes are the major acidic organelles in HEK cells, and that bafilomycin A₁ discharges their H⁺ gradient without affecting their distribution.

Pre-incubation of HEK cells with bafilomycin A₁ caused the increase in [Ca²⁺], evoked by a maximally effective concentration of CCh to increase by 1.7±0.1-fold without affecting the sensitivity to CCh (pEC₅₀=4.3±0.2 and 4.5±0.2 for control and bafilomycin A₁-treated cells, respectively; where pEC₅₀ is the −log of the half-maximally effective concentration; Fig. 1. Inhibition of H⁺ accumulation by acidic organelles exaggerates the increase in [Ca²⁺], evoked by receptors that stimulate phospholipase C. (A) Populations of HEK cells in Ca²⁺-free HBS in 96-well plates were stimulated with a maximal concentration of CCh (1 mM), with (red) or without (black) pre-incubation with bafilomycin A₁ (Baf A₁; 1 μM, 1 h). Baf A₁, an inhibitor of the V-ATPase, dissipates H⁺ gradients across the membranes of acidic organelles and thereby diminishes their ability to accumulate Ca²⁺ by an unidentified pathway that may be a Ca²⁺/H⁺ exchanger (inset). Results are means ± s.e.m. for three wells from a single experiment, each typical of at least three similar experiments. (B) Summary results (means ± s.e.m., n=3 independent experiments) from experiments similar to (A) show concentration-dependent effects of CCh on Ca²⁺ release with and without pre-incubation with bafilomycin A₁. (C) Single-cell analyses in Ca²⁺-free HBS show the peak increase in [Ca²⁺], evoked by CCh (1 mM) with and without pre-incubation with bafilomycin A₁. (D) Intracellular pH (pHᵢ) was measured in populations of HEK cells with (solid bars) or without (open bars) pre-incubation with bafilomycin A₁ (1 μM, 1 h). Baf A₁, vacuolin inhibitor 1 (CI; 10 μM, 10 min) with cathepsin inhibitor 1 (CI; 10 μM, 10 min). Each treatment is paired with its solvent control (0.1–1.1% DMSO). Results are means ± s.e.m. from four coverslips with 50 cells analysed on each. *P<0.05. (E) Intraplasmic pH (pHᵢ) was measured in populations of HEK cells with (solid bars) or without (open bars) pre-incubation with bafilomycin A₁ (1 μM, 1 h). Results are means ± s.e.m. from four coverslips, with 50 cells analysed on each. *P<0.05. (F,G) Single-cell analyses of COS-7 cells in HBS show the peak increase in [Ca²⁺], evoked by ATP (F) and the basal [Ca²⁺], (G) with and without pre-incubation with bafilomycin A₁ (1 μM, 1 h). Results are means ± s.e.m. from three coverslips, with ≥30 cells on each analysed. *P<0.05.
Fig. 1A,B). Bafilomycin A1 also caused a small, but significant ($P=0.0035$), increase in the $[\text{Ca}^{2+}]_i$ of unstimulated cells from $30 \pm 2$ nM to $46 \pm 2$ nM ($n=5$). This modest effect of bafilomycin A1 is probably due largely to basal activity of Ins(1,4,5)P$_3$R since it was abolished by inhibition of PLC (supplementary material Fig. S1E). Analyses of single cells confirmed the results from cell populations. The number of cells responding to a maximal concentration of CCh was similar for control and bafilomycin A1-treated cells ($98 \pm 1\%$ and $96 \pm 3\%$, respectively) and the increase in $[\text{Ca}^{2+}]_i$, was $1.9 \pm 0.3$-fold greater after treatment with bafilomycin A1 (Fig. 1C; supplementary material Table S1).

Stimulation of HEK cells with a submaximal concentration of CCh ($50 \mu$M) evoked sustained Ca$^{2+}$ oscillations, typical of the responses of many cells to physiological stimuli (supplementary material Fig. S4A,B). Bafilomycin A1 had no effect on the number of cells in which CCh evoked Ca$^{2+}$ oscillations ($38 \pm 9\%$ and $50 \pm 6\%$ in control and bafilomycin-treated cells, respectively), but it increased the amplitude of the Ca$^{2+}$ spikes and reduced their frequency (supplementary material Fig. S4C,D).

The affinities of Ca$^{2+}$ indicators and other Ca$^{2+}$-binding sites for Ca$^{2+}$ and of Ins(1,4,5)P$_3$R for Ins(1,4,5)P$_3$ are pH sensitive. We were therefore concerned that bafilomycin A1 might, via effects on cytosolic pH ($\text{pH}_i$), affect the responses observed. Using SNARF-5F to measure $\text{pH}_i$, we established that neither bafilomycin A1 nor any of the other inhibitors used affected $\text{pH}_i$ (Fig. 1D).

These results establish that the increase in $[\text{Ca}^{2+}]_i$, evoked by CCh in HEK cells is potentiated by dissipating the lysosomal H$^+$ gradient. Similar results were obtained with COS-7 cells, where activation of P2Y receptors by ATP evoked Ca$^{2+}$ release from intracellular stores. Bafilomycin A1 caused the peak Ca$^{2+}$ signal evoked by ATP to increase by $1.5 \pm 0.1$ fold (Fig. 1E,F). However, in COS-7 cells, the exaggerated response to ATP occurred without an increase in basal $[\text{Ca}^{2+}]_i$ (Fig. 1G).

Perforating lysosomal membranes potentiates the increase in $[\text{Ca}^{2+}]_i$, evoked by CCh

Glycyl-L-phenylalanine 2-naphthylamide (GPN) allows selective disruption of lysosomes because its cleavage by the lysosomal enzyme, cathepsin C, causes osmotic swelling and thereby perforation of lysosomal membranes (Berg et al., 1994) (Fig. 2A). Treatment of HEK cells with GPN abolished staining with LysoTracker Red without affecting the distribution of LAMP1–mCherry (Fig. 2B) or $\text{pH}_i$ (Fig. 1D). GPN caused the peak Ca$^{2+}$ signal evoked by a maximal concentration of CCh in Ca$^{2+}$-free HEPES-buffered saline (HBS) to increase by $1.5 \pm 0.1$-fold (Fig. 2C), without affecting the sensitivity to CCh (pEC$_{50}$=4.68 $\pm$ 0.03 and 4.64 $\pm$ 0.06 for control and GPN-treated cells; Fig. 2D). GPN also caused a modest increase in basal $[\text{Ca}^{2+}]_i$ ($22 \pm 4$ nM and $42 \pm 5$ nM in control and GPN-treated cells, $P=0.025$; Fig. 2C). To minimize any non-specific effects arising from release of lysosomal contents into the cytosol, we reduced the period of incubation.
with GPN from 30 to 10 min and included cathepsin inhibitor 1 (10 μM), which inhibits many lysosomal proteases, but not the cathepsin C that cleaves GPN (Demuth et al., 1996). The effects of GPN on CCh-evoked Ca^{2+} signals were unaffected by this revised protocol: the peak [Ca^{2+}] signal was increased by 1.6±0.2-fold, with no effect on the sensitivity to CCh (pEC_{50}=4.5±0.1 and 4.3±0.2 in control cells and those treated with GPN and cathepsin inhibitor 1; Fig. 2E).

Bafilomycin A_{1} is expected to inhibit acidification of most acidic organelles, but GPN selectively disrupts lysosomes (Berg et al., 1994). It is therefore significant that GPN abolishes staining by LysoTracker Red (Fig. 2B), confirming that lysosomes are the major acidic organelles in HEK cells. Furthermore, the effects of bafilomycin A_{1} (Fig. 1) and GPN (Fig. 2) on the Ca^{2+} signals evoked by CCh are similar (supplementary material Table S1), confirming that potentiation of Ca^{2+} signals by bafilomycin A_{1} is due to its effects on lysosomes.

**Fusion of lysosomes increases the amplitude of CCh-evoked Ca^{2+} signals**

Vacuolin, by an unknown mechanism, causes fusion of lysosomes (Fig. 3A) (Huynh and Andrews, 2005). In HEK cells transiently expressing LAMP1–mCherry, vacuolin caused lysosomes to fuse (Fig. 3B) without affecting pH_{i} (Fig. 1D). Vacuolin caused a 1.4±0.1-fold increase in the peak [Ca^{2+}]_{i} evoked by CCh without affecting the sensitivity to CCh (pEC_{50}=4.7±0.1 and 4.9±0.1 for control and vacuolin-treated cells; Fig. 3C,D). Single-cell analyses confirmed that vacuolin had no effect on the number of cells that responded to CCh (93±2% and 91±1% for control and vacuolin-treated cells), but increased the amplitude of the CCh-evoked Ca^{2+} signal by 2.0±0.2-fold (Fig. 3E). Vacuolin had no effect on basal [Ca^{2+}]_{i} in either single cells or cell populations (25±4 nM and 30±2 nM in control and vacuolin-treated cells, P=0.31, supplementary material Table S1). The combined effects of maximal concentrations of bafilomycin A_{1} and vacuolin on the peak Ca^{2+} signals evoked by CCh were no different from the effects of either treatment alone (Fig. 3F). The results with vacuolin are important because vacuolin is not expected to affect lysosomal ion transport mechanisms, but instead to change the surface area to volume ratio of lysosomes and thereby to perturb interactions with other organelles.

Because some, though not all, treatments used to manipulate lysosomal activity caused modest increases in basal [Ca^{2+}]_{i} (Fig. 1E; supplementary material Table S1), we were concerned that potentiated responses to Ins(1,4,5)P_{3} might result from enhanced Ca^{2+} uptake by the ER. We therefore used a novel low-affinity Ca^{2+} indicator targeted to the ER, CatchER (Tang et al., 2011), to measure directly the free [Ca^{2+}]_{i} within the ER of COS-7 cells. These are better suited than HEK cells for these studies because COS-7 cells are larger, flatter and their ER is more easily identified. The results demonstrate that under conditions where bafilomycin A_{1} increased the peak [Ca^{2+}]_{i} evoked by ATP, it had no effect on the free [Ca^{2+}]_{i} within the ER (supplementary material Fig. S5). The larger increases in [Ca^{2+}]_{i} evoked by PLC-linked receptors in the presence of bafilomycin A_{1} are not therefore due to increased loading of the ER with Ca^{2+}.

Three means of perturbing the behaviour of lysosomes, inhibition of the V-ATPase (Fig. 1), disruption of lysosomal membranes (Fig. 2) or modification of lysosomal morphology (Fig. 3), indistinguishably and non-additively (Fig. 3F) potentiate
the Ca\(^{2+}\) signals evoked by CCh. Because CCh-evoked Ca\(^{2+}\) signals are entirely mediated by Ins(1,4,5)P\(_3\) (supplementary material Fig. S1), we examined the effect of bafilomycin A\(_1\) on the Ca\(^{2+}\) signals evoked by directly activating Ins(1,4,5)P\(_3\).

Lysosomes accumulate Ca\(^{2+}\) released by direct activation of Ins(1,4,5)P\(_3\) receptors

Flash photolysis of caged Ins(1,4,5)P\(_3\) (ci-Ins(1,4,5)P\(_3\)) non-disruptively loaded into cells allows Ins(1,4,5)P\(_3\) to be delivered directly to the cytosol (Dakin and Li, 2007). Photolysis of ci-Ins(1,4,5)P\(_3\) caused transient increases in [Ca\(^{2+}\)], the amplitudes of which varied between cells (Fig. 4A), probably reflecting differences between cells in loading and/or de-esterification of ci-Ins(1,4,5)P\(_3\) to be delivered directly to the cytosol. This confirms that when Ins(1,4,5)P\(_3\);R are directly activated by Ins(1,4,5)P\(_3\), Ca\(^{2+}\) signals are potentiated by inhibition of the lysosomal V-ATPase. The increase in [Ca\(^{2+}\)], evoked by photolysis of ci-Ins(1,4,5)P\(_3\) recovered with mono-exponential kinetics (Fig. 4B), but recovery was much slower (P=0.029) in cells treated with bafilomycin A\(_1\) (half-time, \(t_{1/2}=45.6\pm7.8\) s) than in control cells (\(t_{1/2}=18.9\pm1.7\) s; Fig. 4D). Bafilomycin A\(_1\) also slowed recovery from CCh-evoked Ca\(^{2+}\) signals (supplementary material Fig. S6). The sustained effects of bafilomycin A\(_1\) on the Ca\(^{2+}\) signals evoked by CCh or photolysis of ci-Ins(1,4,5)P\(_3\) probably reflect cycling of Ca\(^{2+}\) through the ER fuelled by the activity of the Ca\(^{2+}\) pump of the ER (SR/ER Ca\(^{2+}\)-ATPase; SERCA) and sustained activity of Ins(1,4,5)P\(_3\);R. These results confirm that bafilomycin A\(_1\) potentiates Ins(1,4,5)P\(_3\)- or CCh-evoked Ca\(^{2+}\) signals by inhibiting removal of Ca\(^{2+}\) from the cytosol through lysosomal uptake systems.

Bafilomycin A\(_1\) also potentiated the transient Ca\(^{2+}\) signals evoked by inhibiting the SERCA with thapsigargin in nominally Ca\(^{2+}\)-free HBS (Fig. 5A,B). Similar results (1.9±0.2-fold increase in the peak Ca\(^{2+}\) signal, n=3) were obtained when cyclopiazonic acid (CPA) was used to inhibit the SERCA (supplementary material Fig. S7). The route by which Ca\(^{2+}\) leaks from the ER is undefined, but it may include contributions from translocons (Lang et al., 2011) and Ins(1,4,5)P\(_3\);R (supplementary material Fig. S1). Both channels have large Ca\(^{2+}\) conductances that might be expected to generate the large local increases in [Ca\(^{2+}\)] that we suggest are required for lysosomal Ca\(^{2+}\) uptake.

Ca\(^{2+}\) signals evoked by store-operated Ca\(^{2+}\) entry are unaffected by lysosomes

Our results so far demonstrate that Ca\(^{2+}\) released from the ER via Ins(1,4,5)P\(_3\);R or leak pathways (after addition of CPA or thapsigargin) can be accumulated by lysosomes. We next assessed whether Ca\(^{2+}\) entering the cell across the plasma membrane via SOCE is also accumulated by lysosomes. Restoration of extracellular Ca\(^{2+}\) to cells treated with thapsigargin in nominally Ca\(^{2+}\)-free HBS evoked sustained increases in [Ca\(^{2+}\)] (Fig. 5C). This is consistent with abundant evidence for SOCE in HEK cells (Parekh and Putney, 2005). Manipulating the extracellular [Ca\(^{2+}\)] allowed the amplitude of the global increase in [Ca\(^{2+}\)], evoked by SOCE to match and exceed (Fig. 5D) the peak Ca\(^{2+}\) signals evoked by CCh in Ca\(^{2+}\)-free HBS (Fig. 1B). Nevertheless, the Ca\(^{2+}\) signals evoked by SOCE were entirely insensitive to bafilomycin A\(_1\) (Fig. 5C,D). Similar results were obtained when CPA (100 \(\mu\)M, 15 min) was used to evoke SOCE: the peak Ca\(^{2+}\) signals evoked by addition of HBS containing 30 mM Ca\(^{2+}\) were 180±40 nM and 203±48 nM in control and bafilomycin A\(_1\)-treated cells, respectively (supplementary material Fig. S7). A possible concern is that Ca\(^{2+}\) released from the ER after addition of thapsigargin or CPA is accumulated by lysosomes (Fig. 5A,B) and might thereby limit their capacity to accumulate further Ca\(^{2+}\) entering the cell via SOCE. To address this issue, we used a membrane-permeant low-affinity Ca\(^{2+}\) buffer, TPEN (N,N',N'-tetraakis(2-pyridylmethyl)-1,2-ethylenediamine), to reduce the free [Ca\(^{2+}\)] within the ER and so activate SOCE without causing Ca\(^{2+}\) release (Hofer et al., 1998) (supplementary material Fig. S8). Restoration of extracellular Ca\(^{2+}\)
to HEK cells treated with TPEN (100 μM, 2 min) stimulated SOCE-mediated increases in [Ca^{2+}]; that were insensitive to bafilomycin A1 (Fig. 5E). CCh-evoked Ca^{2+} release in these TPEN-treated cells was potentiated by bafilomycin A1 (1 μM, 1 h). (B) Summary results show effects of bafilomycin A1 (1 μM, 1 h) on the peak increase in [Ca^{2+}], evoked by thapsigargin (1 μM) in nominally Ca^{2+}-free HBS. Results are means ± s.e.m. from three independent experiments. (C) Restoration of extracellular Ca^{2+} (30 mM) to cells pre-treated with thapsigargin (1 μM, 15 min) in nominally Ca^{2+}-free HBS to deplete intracellular Ca^{2+} stores evokes SOCE. The response is indistinguishable in control cells and cells treated with bafilomycin A1 (1 μM, 1 h). (D) SOCE after restoration of different concentrations of extracellular Ca^{2+} ([Ca^{2+}]_{e}) to control and bafilomycin A1-treated cells (1 μM, 1 h). Results are means ± s.e.m. from three independent experiments. (E) TPEN (100 μM, 2 min) in nominally Ca^{2+}-free HBS was used to reduce the free [Ca^{2+}] within the ER before restoration of the indicated concentrations of extracellular Ca^{2+} to control or bafilomycin A1-treated cells (1 μM, 1 h). Results show the peak increase in [Ca^{2+}], detected within 60 s after restoration of extracellular Ca^{2+}. (F) Peak increase in [Ca^{2+}], evoked by CCh (1 mM) in the presence of TPEN (100 μM, 2 min) with and without pre-incubation with bafilomycin A1 (1 μM, 1 h). Results are means ± s.e.m. from three (E) or four (F) independent experiments. Examples of traces from which the summary results shown in E and F were derived are shown in supplementary material Fig. S8. *P<0.05.

**Fig. 5. Disruption of lysosomal Ca^{2+} uptake with bafilomycin A1 does not affect SOCE.** (A) Transient increase in [Ca^{2+}], evoked by thapsigargin (1 μM, solid bar) in HEK cells in nominally Ca^{2+}-free HBS in control and bafilomycin A1-treated cells (Baf A1; 1 μM, 1 h). (B) Summary results show effects of bafilomycin A1 (1 μM, 1 h) on the peak increase in [Ca^{2+}], evoked by thapsigargin (1 μM) in nominally Ca^{2+}-free HBS. Results are means ± s.e.m. from three independent experiments. (C) Restoration of extracellular Ca^{2+} to cells pre-treated with thapsigargin (1 μM, 15 min) in nominally Ca^{2+}-free HBS to deplete intracellular Ca^{2+} stores evokes SOCE. The response is indistinguishable in control cells and cells treated with bafilomycin A1 (1 μM, 1 h). (D) SOCE after restoration of different concentrations of extracellular Ca^{2+} ([Ca^{2+}]_{e}) to control and bafilomycin A1-treated cells (1 μM, 1 h). Results are means ± s.e.m. from three independent experiments. (E) TPEN (100 μM, 2 min) in nominally Ca^{2+}-free HBS was used to reduce the free [Ca^{2+}] within the ER before restoration of the indicated concentrations of extracellular Ca^{2+} to control or bafilomycin A1-treated cells (1 μM, 1 h). Results show the peak increase in [Ca^{2+}], detected within 60 s after restoration of extracellular Ca^{2+}. (F) Peak increase in [Ca^{2+}], evoked by CCh (1 mM) in the presence of TPEN (100 μM, 2 min) with and without pre-incubation with bafilomycin A1 (1 μM, 1 h). Results are means ± s.e.m. from three (E) or four (F) independent experiments. Examples of traces from which the summary results shown in E and F were derived are shown in supplementary material Fig. S8. *P<0.05.

**CCh increases the luminal pH of lysosomes**

So far, our analyses of the contribution of lysosomes to shaping Ins(1,4,5)P_{3}-evoked Ca^{2+} signals have relied on measurements of [Ca^{2+}], Complementary analyses of free [Ca^{2+}] within lysosomes ([Ca^{2+}]_{ly}) are more challenging. Indicators can be directed to the lysosome lumen by endocytosis, but their affinity for Ca^{2+} is reduced by the low lysosomal pH (~pH 4) (Christensen et al., 2002; Lloyd-Evans et al., 2008). We assessed the effects of CCh on lysosomal pH (pH_{ly}) and [Ca^{2+}]_{ly} using pairs of endocytosed dextran-conjugated indicators: an inert marker (Texas Red, TR) and either Oregon Green (OG, to measure pH) or Oregon Green BAPTA (OGB, to measure [Ca^{2+}]; Fig. 6).

In populations of HEK cells, CCh (1 mM) evoked a sustained increase in OG fluorescence, without affecting TR fluorescence (Fig. 6B,C). This demonstrates that CCh caused an increase in pH_{ly}. Parallel measurements of cytosolic pH (pH_{i}) established that in both the presence and absence of extracellular Ca^{2+}, CCh evoked a slow and very modest decrease in pH_{i} (Fig. 6D). The effect was statistically significant (P=0.009) only for cells stimulated with CCh for 5 minutes in the presence of extracellular Ca^{2+}, when pH_{i} fell from 7.42±0.02 to 7.34±0.01 (Fig. 6D). These results demonstrate that under conditions where pH_{i} is either stable or very modestly reduced (Fig. 6D), CCh evokes an increase in pH_{ly} (Fig. 6B,C). The effect of CCh on pH_{ly} cannot therefore be mediated by changes in pH_{i}. It is more likely to be caused by CCh-evoked Ca^{2+} signals and subsequent lysosomal Ca^{2+} uptake by Ca^{2+}/H^{+} exchange. This interpretation is consistent with evidence that addition of Ca^{2+} to isolated acidic organelles increases luminal pH (Morgan et al., 2011).

It is more difficult to measure directly the free [Ca^{2+}] within lysosomes because the Ca^{2+} affinity of OGB, like all other Ca^{2+} indicators, is pH sensitive. Nevertheless, in similar analyses using TR and OGB, CCh selectively increased the fluorescence of the lysosomal Ca^{2+} indicator (OGB; Fig. 6E-G). These direct measurements of pH_{ly} and indirect measurement of [Ca^{2+}]_{ly} concur with the analyses of [Ca^{2+}], (Figs 1–5) by suggesting that lysosomes sequester Ca^{2+} released from intracellular stores.
Lysosomes are motile and associated with ER

We used total internal reflection fluorescence microscopy (TIRFM) to examine the distribution of ER and lysosomes in COS-7 cells by labelling ER with GFP–Ins(1,4,5)P$_3$R1, GFP–Ins(1,4,5)P$_3$R3 or GFP–ER, all of which colocalised with co-transfected SERCA–mCherry (supplementary material Fig. S9). Lysosomes, identified with either LysoTracker Red or LAMP1–mCherry, were closely associated with the ER (Fig. 7A): 79±3% ($n=7$ cells) of lysosomes were close enough to the ER that their separation could not be resolved by TIRFM. As reported for other cells (Matteoni and Kreis, 1987), lysosomes were remarkably motile within COS-7 cells (supplementary material Movie 1).

Video-rate imaging of COS-7 cells co-transfected with GFP–ER and LAMP1–mCherry allowed tracking of individual lysosomes and showed that lysosomes maintain their association with the ER over prolonged periods (often for the entire 2-minute recording) as they move around the cell (Fig. 7B; supplementary material Movie 1). This movement often occurred along ER tubules, and occasionally lysosomes localised at the tips of ER tubules and moved concomitantly with ER tubule extension, further supporting a close spatial relationship between ER and lysosomes.

Discussion

Lysosomes accumulate Ca$^{2+}$ released from the ER

Disruption of lysosomal Ca$^{2+}$ uptake mechanisms by preventing luminal acidification (Figs 1, 4) or by perforation of lysosomal membranes (Fig. 2), or changing the morphology of lysosomes and so their relationships with other organelles (Fig. 3) exaggerates the cytosolic Ca$^{2+}$ signals evoked by Ins(1,4,5)P$_3$. This occurs whether Ins(1,4,5)P$_3$ is delivered by activation of endogenous receptors in the PM (Figs 1–3) or by photolysis of ci-Ins(1,4,5)P$_3$ (Fig. 4), and it occurs in both HEK and COS-7 cells. Despite the increased Ca$^{2+}$ signals, there was no change in CCh sensitivity, suggesting that the treatments affected neither the intracellular concentrations of Ins(1,4,5)P$_3$ nor the sensitivity of Ins(1,4,5)P$_3$R. The decrease in frequency, but increase in amplitude, of CCh-evoked Ca$^{2+}$ spikes after bafilomycin A$_1$ treatment (supplementary material Fig. S4) is also inconsistent with a simple increase in either Ins(1,4,5)P$_3$ concentration or Ins(1,4,5)P$_3$R sensitivity because either would be expected to increase the frequency of Ca$^{2+}$ oscillations. Furthermore, CCh-evoked Ca$^{2+}$ release is accompanied by a sustained increase in lysosomal pH and perhaps lysosomal [Ca$^{2+}$] (Fig. 6), consistent with sequestration of Ca$^{2+}$ by a lysosomal Ca$^{2+}$/H$^+$ exchanger.
In HEK cells, inhibition of lysosomal Ca\(^{2+}\) accumulation with bafilomycin A\(_1\) or GPN was accompanied by a small (<20 nM) increase in basal [Ca\(^{2+}\)]. Nevertheless, we were concerned that these small increases in basal [Ca\(^{2+}\)] might contribute to the increased amplitude of the Ins\((1,4,5)P_3\)-evoked Ca\(^{2+}\) signals by enhancing loading of Ca\(^{2+}\) stores or by sensitization of Ins\((1,4,5)P_3R\). The latter is unlikely because none of the treatments affected the sensitivity to CCh (pEC\(_{50}\); Fig. 1B; Fig. 2D,E; Fig 3D; and see above) and they potentiated the Ca\(^{2+}\) signals evoked by Ca\(^{2+}\) leaks from the ER (Fig. 5A; supplementary material Fig. S7A).

Additional evidence establishes that enhanced Ca\(^{2+}\) loading of the ER is not the explanation. First, bafilomycin A\(_1\) enhanced the increase in [Ca\(^{2+}\)], evoked by ATP in COS-7 cells without affecting basal [Ca\(^{2+}\)] (Fig. 1E–G) and vacuolin mimicked the effects of bafilomycin A\(_1\) and GPN on CCh-evoked Ca\(^{2+}\) signals in HEK cells without affecting basal [Ca\(^{2+}\)] (supplementary material Table S1). Second, direct measurement of luminal [Ca\(^{2+}\)] within the ER demonstrated that bafilomycin A\(_1\) did not affect Ca\(^{2+}\) uptake by the ER (supplementary material Fig. S5), and buffering ER Ca\(^{2+}\) with TPEN did not prevent bafilomycin A\(_1\) from potentiating CCh-evoked Ca\(^{2+}\) signals (Fig. 5F). Third, after CCh stimulation (supplementary material Fig. S6) or flash photolysis of ci-Ins\((1,4,5)P_3\) (Fig. 4), [Ca\(^{2+}\)] recovered more slowly in bafilomycin A\(_1\)-treated cells, confirming that bafilomycin A\(_1\) inhibits Ca\(^{2+}\) removal from the cytosol. Finally, CCh increased pH\(_{ly}\) and perhaps [Ca\(^{2+}\)]\(_{ly}\) in the absence of inhibitors (Fig. 6), suggesting that Ca\(^{2+}\) released via Ins\((1,4,5)P_3R\) is sequestered by lysosomes.

We conclude that the increased amplitude of the Ca\(^{2+}\) signals evoked by Ins\((1,4,5)P_3\) after disruption of lysosomes is due to their diminished ability to sequester Ca\(^{2+}\) released from the ER. These observations are consistent with studies where thapsigargin evoked larger cytosolic Ca\(^{2+}\) signals (Pereira et al., 2010), and lysosomal Ca\(^{2+}\) uptake was diminished (Lloyd-Evans et al., 2008) in cells with lysosomal storage disorders.

**ER and lysosomes are intimately associated**

CCh evokes Ca\(^{2+}\) signals in HEK cells that are initiated by Ins\((1,4,5)P_3R\) and then sustained by SOCE. Although many lysosomes were present in the TIRFM field, suggesting that they lie within ~100 nm of the PM, they did not sequester Ca\(^{2+}\) entering via SOCE even when the global increases in [Ca\(^{2+}\)] exceeded those evoked by Ins\((1,4,5)P_3\). This demonstrates that lysosomes selectively sequester Ca\(^{2+}\) released from the ER. There are several implications of this conclusion. First, the global elevations in [Ca\(^{2+}\)] that occur as Ca\(^{2+}\) diffuses away from open channels are insufficient to allow significant stimulation of Ca\(^{2+}\) uptake by lysosomes. This suggests that the lysosomal uptake system responsible for these effects has a low affinity for Ca\(^{2+}\). The sparse studies of Ca\(^{2+}\) uptake by isolated lysosomes differ enormously in their estimates of the affinity (K\(_m\)) for Ca\(^{2+}\): 108 nM and 5.7 mM for lysosomes isolated from neutrophils (Styrt et al., 1988) and fibroblasts (Lemons and Thoene, 1991), respectively. The significance of the latter study is unclear because the incubations excluded ATP. Second, the lysosomal Ca\(^{2+}\) uptake system responsible for the effects observed in our study is unlikely to be a Ca\(^{2+}\) pump because pumps are too slow (e.g. turnover number ~10 s\(^{-1}\) for SERCA) (Lytton et al., 1992) to sequester Ca\(^{2+}\) effectively before it diffuses away from an open Ins\((1,4,5)P_3R\) that probably conducts ~500,000 Ca\(^{2+}\) s\(^{-1}\) (Vais...
Ca$^{2+}$ sequestration by lysosomes

ER–organelle junctions: a recurring theme in Ca$^{2+}$ signalling

We have shown that dynamic lysosomes and ER are intimately associated (Fig. 7; supplementary material Movie 1), allowing lysosomes selectively to accumulate Ca$^{2+}$ released from the ER.

The reciprocal relationship is also important because in many cells NAADP-evoked Ca$^{2+}$ release from lysosomes triggers Ca$^{2+}$ release from the ER by Ca$^{2+}$-induced Ca$^{2+}$ release via Ins(1,4,5)P$_{2}$R (Calcraft et al., 2009) or RyR (Brailoiu et al., 2010; Cancela et al., 1999; Kinnear et al., 2008; Lee et al., 1997). Lysosome–ER junctions are reminiscent of those between mitochondria and ER (Fig. 8). The latter are maintained by specific tethering proteins between dynamic organelles (Csordás et al., 2006) and they allow mitochondrial Ca$^{2+}$ uptake by the low-affinity Ca$^{2+}$ uniporter, rapidly sequestering Ca$^{2+}$ (Olson et al., 2010), while Ca$^{2+}$ provided by ER Ca$^{2+}$ channels allows mitochondrial Ca$^{2+}$ uptake to regulate oxidative phosphorylation (Jouaville et al., 1999), apoptosis (Szalai et al., 1999) and mitochondrial motility (Yi et al., 2004). We suggest a similar bi-directional interplay between ER and lysosomes (Fig. 8). Ca$^{2+}$ release via TPC2 can trigger Ca$^{2+}$ release via RyR or Ins(1,4,5)P$_{2}$R in the ER (Brailoiu et al., 2010; Calcraft et al., 2009; Kinnear et al., 2008), and Ca$^{2+}$ release via Ins(1,4,5)P$_{2}$R is selectively accumulated by lysosomes. The latter may regulate the behaviour of lysosomes by increasing lysosomal pH; by priming TPC2, which appears to shape the Ca$^{2+}$ oscillations typically evoked by physiological stimuli, and by analogy with mitochondria (Olson et al., 2010) lysosomes may modulate feedback regulation of Ins(1,4,5)P$_{2}$R gating (Fig. 8).

Fig. 8. Functional Ca$^{2+}$ junctions: similarities between lysosomes and mitochondria. Mitochondria, via the low-affinity Ca$^{2+}$ uniporter, rapidly accumulate Ca$^{2+}$ when exposed to high local [Ca$^{2+}$], around the mouth of open Ca$^{2+}$ channels, such as Ins(1,4,5)P$_{2}$R (IP$_{2}$R). Our results suggest that lysosomes can also selectively accumulate Ca$^{2+}$, presumably via a low-affinity uptake pathway, from within microdomains of substantially increased [Ca$^{2+}$], around open Ins(1,4,5)P$_{2}$R. We have shown that lysosomes can thereby shape the Ca$^{2+}$ signals evoked by Ins(1,4,5)P$_{2}$R. Mitochondria likewise regulate cytosolic Ca$^{2+}$ signals both locally (by intercepting Ca$^{2+}$-mediated interactions between Ins(1,4,5)P$_{2}$R and more globally. The reverse interaction, where Ca$^{2+}$ release from lysosomes via TPC2, selectively stimulates Ins(1,4,5)P$_{2}$R or RyR by Ca$^{2+}$-induced Ca$^{2+}$ release, seems to have no obvious parallel with mitochondria. However, Ca$^{2+}$ uptake by mitochondria triggered by opening of Ins(1,4,5)P$_{2}$R regulates mitochondrial activity. We speculate that Ca$^{2+}$ uptake by lysosomes may also regulate their activity by, for example, regulating lysosomal pH and thereby enzyme activity, endolysosomal trafficking and priming TPC2 to respond to NAADP.

Materials and Methods

Materials

Culture media, Lipofectamine 2000, LysoTracker Red DND-99, SNARF-5F/AM, fluo-4/AM, fura-2/AM, dextran-conjugated Texas Red (Mw, 70,000), dextran-conjugated Oregon Green BAPTA 488 (Mw, 10,000), dextran-conjugated Oregon Green 488 (Mw, 10,000) and the Ca$^{2+}$ standard solutions used to calibrate fura-2 fluorescence signals to [Ca$^{2+}$], were from Invitrogen (Paisley, UK). Cell culture plastics and 96-well assay plates were from MatTek (Ashland, USA) or PAA Laboratories (Yeovil, UK). U73122 [1S-(17β)-3-methoxyxestra-1,3,5(10)-trien-17-y]aminooxycyl-1H-pyrole-2,5-dione] and CPA were from Tocris (Bristol, UK). Bafilomycin A$_{1}$ was from AG Scientific (California, USA) or Fluorochem (Hadfield, UK). Glycyl-L-phenylalanine 2-naphthylamide (GPN) was from Bachem (St. Helens, UK). Cathepsin inhibitor 1 was from Calbiochem (Nottingham, UK). BAPTA was from Mol Evolut (Dorset, UK). CCh, ATP, DMSO, foetal bovine serum, poly-L-lysine and vacuolin were from Sigma-Aldrich (Poole, UK). Ionomycin was from MerkEurolab (Nottingham, UK). Thapsigargin was from Alexon (Jerusalem, Israel). Caged cell-permeant Ins(1,4,5)P$_{2}$ (ci-Ins(1,4,5)P$_{2}$);PM) was from StChem (Bremen,
Plasmids

Plasmids encoding LAMP1-mCherry and LAMP1-GFP were made by transferring the LAMP1 fragment from LAMP1-tdTomato (a gift from T. Woz´niak et al., 2009). A plasmid encoding SERCA type-1 (a gift from T. Ryan, Manchester, UK) (Woz´niak et al., 2009). A plasmid encoding SERCA type-1 (a gift from T. Ryan, Manchester, UK) was from Tovey et al., 2008) into pmCherry N1 or pAcGFP-N1 (Clontech, Mountain View, USA) according to the manufacturer's instructions with 1 μg DNA. A plasmid encoding the ER-located Ca2+-sensor CatchER (Tang et al., 2011). COS-7 cells in 35-mm imaging dishes were transfected with the CatchER coding sequence in pmDNA3.1 (2 μg/well) using Lipofectamine 2000 (1:1, DNA/Lipofectamine). After 48 h, cells were imaged using an Olympus IX81 TIRF microscope with 60× or 150× TIRFM objectives. In time-lapse experiments, images were captured using wide-field illumination (488 nm, 200-ms exposure time) with a 60× TIRF objective and an Andor iXon 897 EMCCD camera. Control cells were used to correct images for photobleaching (~15% over 1 h).

Flash photolysis of caged Ins(1,4,5)P3

HEK cells grown on poly-L-lysine-coated, glass-bottomed culture dishes were used there was no significant crosstalk between green and red channels. Images were acquired at 1-second intervals with an Andor iXon 897 camera (512×512 pixels) and analysed using Cell R software (Olympus, Milton Keynes, UK). For these measurements of [Ca2+]i, using a non-ratiometric indicator (fluor-4), responses are reported as F/Fo where Fo is the average fluorescence intensity recorded from a ROI immediately before flash photolysis, and F is the fluorescence intensity from the same region after the flash.

Measurement of cytosolic pH

Cytosolic pH (pHi) was measured using the fluorescent pH-sensitive indicator, SNARF-5F (Liu et al., 2001). Cells in 96-well plates were incubated with SNARF-5F/AM (2 μM, 30 min), washed with HBSS, and fluorescence (excitation at 561 nm, and emission at 580 and 640 nm) was measured using a FlexStation fluorescence plate reader. Fluorescence ratios (F580/F640) were calibrated to pHi using standard pH solutions (Owen, 1992).

Total internal reflection fluorescence microscopy

For TIRFM, cells on poly-L-lysine-coated, glass-bottomed culture dishes were imaged using an Olympus IX81 microscope with 150×/1.45 NA or 60×/1.45 NA TIRF objectives. For staining of lysosomes, cells were incubated with LysoTracker Red DND-99 (50 nM, 1 h, 20°C). Cells were illuminated with 488 nm (for GFP) or 561 nm (for LAMP1–mCherry) laser diode-based solid-state lasers, and images were acquired with an Andor iXon 897 EMCCD camera. With the filters used there was no significant crosstalk between green and red channels. Images were processed using Cell R software (Olympus, Milton Keynes, UK).

Analysis

Concentration-effect relationships for each experiment were individually fitted to Hill equations using non-linear curve-fitting (GraphPad Prism, version 5) and the results obtained from each (pEC50, Hill coefficient h, maximal response) were pooled for statistical analysis and presentation. Rates of recovery of [Ca2+]i after flash photolysis of ci-Ins(1,4,5)P3 were determined by fitting mono-exponential decay equations (GraphPad Prism, version 5) to the averaged responses of all cells within the field exposed to the UV-flash (30–70 cells/field). At least three such fields were analysed for each coverslip and the half-times (t1/2) were pooled to produce a single value for each coverslip. Rates of recovery of [Ca2+]i, after stimulation of cell populations with CCh were determined by fitting bi-exponential decay equations (GraphPad Prism, version 5). Each determination comprises the average response from at least three wells in a single experiment.

For quantitative analyses of the colocalisation of two fluorophores, Cell R software was used to correct for background fluorescence using an area outside the cell and then to define ROIs (~200–400 μm) within the peripheral cytoplasm that excluded the nucleus and perinuclear area. For each ROI examined (Fig. 7; supplementary material Figs S2, S9), there was a statistically significant (P<0.05) colocalisation of fluorophores defined using the Colocalisation Analysis/Colocalisation Test plugin (ImageJ). This applies the Costes randomization method with 100 iterations and ignores pixels in which there is no fluorescence colocalisation (Olympus, Milton Keynes, UK). All records were corrected for background fluorescence determined under identical conditions from cells that had not been loaded with indicators. Fluorescence changes from defined regions of interest (ROI) were then expressed as F/Fo, where Fo and F denote the average fluorescence within the ROI at the start of the experiment (Fo) and at each time point (F).

Measurement of luminal free [Ca2+]i in the ER

The luminal free [Ca2+]i of the ER was measured using an ER-targeted Ca2+-sensor CatchER (Tang et al., 2011). COS-7 cells in 35-mm imaging dishes were transfected with the CatchER coding sequence in pcDNA3.1 (2 μg/well) using Lipofectamine 2000 (1:1, DNA/Lipofectamine). After 48 h, cells were imaged using an Olympus IX81 TIRF microscope with 60× or 150× TIRFM objectives. In time-lapse experiments, images were captured using wide-field illumination (488 nm, 200-ms exposure time) with a 60× TIRF objective and an Andor iXon 897 EMCCD camera. Control cells were used to correct images for photobleaching (~15% over 1 h).

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from either fluorophore (Costes et al., 2004). Colocalisation was then quantified using the Colocalisation Analysis/Colocalisation Threshold plugin (ImageJ). This was applied to threshold images and then calculate Pearson’s correlation coefficient \( r_{\text{Pearson}} \), ignoring pixels with intensities below threshold. 

\[
\text{Pearson’s correlation coefficient } = \frac{\sum (G_i - \text{mean}(G)) (R_i - \text{mean}(R))}{\sqrt{\sum (G_i - \text{mean}(G))^2 \sum (R_i - \text{mean}(R))^2}},
\]

where \( G_i \) and \( R_i \) are the intensities of individual green and red pixels respectively, and \( \text{mean}(G) \) and \( \text{mean}(R) \) are the mean intensities of red and green pixels. \( r_{\text{Pearson}} \approx 1 \) denotes perfect colocalisation.

Student's t-test or 1-way ANOVA was used for statistical analyses with \( P<0.05 \) considered significant. All statistical analyses were performed on raw data, although for clarity of presentation some results are shown normalized and with statistical significance shown for the underlying raw data (Fig. 1C,F; Fig. 3E; Fig. 4C).

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Supplementary material
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Fig. S1. CCh-evoked Ca²⁺ release in HEK cells is entirely mediated by Ins(1,4,5)P₃ (IP₃) receptors. (A) Intracellular targets of the inhibitors used. (B-D) Peak increases in [Ca²⁺]ᵢ recorded from populations of HEK cells in Ca²⁺-free HBS after stimulation with CCh alone or after pre-incubation with ryanodine (20 μM, 15 min, B) to inhibit RyR, NED 19 (10 μM, 15 min, C) to inhibit TPC, or U73122 (10 μM, 2 min, D) to inhibit PLC. Results are means ± s.e.m. from 3 experiments. (E) Basal [Ca²⁺]ᵢ in populations of control HEK cells in nominally Ca²⁺-free HBS or after treatment with bafilomycin A₁ (Baf A₁; 1 μM, 1 h), U73122 (10 μM, 2 min) or both. Results (means ± s.e.m. from 5 independent experiments) show that U73122 abolishes the effects of bafilomycin A₁ on basal [Ca²⁺]ᵢ. *P<0.05.
**Fig. S2.** Colocalisation of LysoTracker Red with LAMP1-GFP in HEK cells. (A,B) Wide-field (A) and TIRFM (B) images of HEK cells transfected with LAMP1-GFP and stained with LysoTracker Red (10 nM, 2 h). Scale bar applies to all images. Colocalisation of LysoTracker Red with LAMP1-GFP in HEK cells was statistically significant ($P<0.05$) with $R_{\text{coloc}}$ of 0.77±0.02 (n≥11 cells from 3 independently transfected coverslips).

**Fig. S3.** Effects of bafilomycin A$_1$ on lysosomal pH and distribution of lysosomes in HEK cells. TIRFM images of HEK cells stained with LysoTracker Red (50 nM, 1 h; upper panels) or transiently expressing LAMP1-mCherry (lower panels) with or without pre-incubation with bafilomycin A$_1$ (Baf A$_1$, 1 μM, 1 h, and during incubation with LysoTracker Red). Differential interference contrast (DIC) images are shown alongside the equivalent fluorescent images. Images are typical of results from 3 coverslips. Scale bars: 20 μm in all images. Fig. S2 shows that LysoTracker Red staining colocalises with LAMP1.
Fig. S4. Bafilomycin A₁ increases the amplitude and reduces the frequency of CCh-evoked Ca²⁺ oscillations. (A,B) Cells were stimulated with CCh (50 μM) in HBS alone (A) or after treatment with bafilomycin A₁ (1 μM, 1 h) (B), and cytosolic Ca²⁺ signals were recorded from individual cells. Three traces are shown for each condition to illustrate the variability between cells. (C,D) Summary data show the amplitudes of successive Ca²⁺ spikes (C) and the number of spikes recorded in each successive 5-min interval (D). Results are means ± s.e.m. from 3 independent experiments with at least 60 cells analysed in each.
Fig. S5. Bafilomycin A₁ does not affect the luminal free [Ca²⁺] of the ER. (A) Wide-field and TIRFM images show expression of CatchER in the ER of COS-7 cells. The scale bar applies to both images. (B) Wide-field images of COS-7 cells expressing CatchER imaged at the times shown (in mins) after addition of bafilomycin A₁ (1 μM). (C) Summary data from experiments similar to those in B show CatchER fluorescence (ΔF/F₀) from individual cells with and without bafilomycin A₁ treatment. Results are means ± s.e.m. for 16 (control) or 18 (bafilomycin A₁) cells from 4-5 independent experiments. (D) COS-7 cells expressing CatchER were exposed to CPA (20 μM) to inhibit SERCA and reduce luminal [Ca²⁺], or to ionomycin (1 μM) and extracellular Ca²⁺ (10 mM) to increase luminal Ca²⁺. Images are shown before and 5 min after additions. (E) Traces from experiments similar to those in D and corrected for slow bleaching of the indicator show the change in CatchER fluorescence after addition (solid bar) of CPA or ionomycin with Ca²⁺. Results show averages from 3-6 cells on a single coverslip and are representative of at least 4 independent experiments. The results in D and E show that steady-state loading of ER with Ca²⁺ does not saturate the indicator.
Fig. S6. Bafilomycin A₁ slows recovery from CCh-evoked Ca²⁺ signals. (A,B) Typical results from populations of HEK cells stimulated with CCh (1 mM) alone or after treatment with bafilomycin A₁ in HBS (A) or Ca²⁺-free HBS (B) showing the time course of the Ca²⁺ signals after the initial peak. The decaying phase of CCh-evoked Ca²⁺ signals was more complex than after photolysis of caged Ins(1,4,5)P₃ (Fig. 4B), probably reflecting contributions from heterogeneity of responses within a cell population and from additional steps in the more complex sequence between activation of cell-surface receptors and Ins(1,4,5)P₃-evoked Ca²⁺ release. The results were adequately described by the sum of two exponential equations. Results show typical results (n=1) showing means ± s.e.m. from 6 wells within a single experiment. (C,D) Summary results from the bi-exponential curve-fits show half-times (t₁/₂) for the fast (C) and slow (D) components of recovery. Results are means ± s.e.m. from 16 independent experiments. *P<0.05.
Fig. S7. Ca\(^{2+}\) signals evoked by SOCE triggered by depleting stores with cyclopiazonic acid are unaffected by bafilomycin A\(_1\). (A) HEK cells in nominally Ca\(^{2+}\)-free HBS with and without bafilomycin A\(_1\) treatment (Baf A\(_1\), 1 \(\mu\)M, 1 h) were stimulated with cyclopiazonic acid to inhibit SERCA (CPA, 100 \(\mu\)M, solid bar). Results show means ± s.e.m. from triplicate determinations within a single experiment. (B) Summary results (means ± s.e.m. from 3 independent experiments) show the effects of bafilomycin A\(_1\) on the peak increase in \([\text{Ca}^{2+}]_i\) evoked by CPA. (C) Extracellular Ca\(^{2+}\) (30 mM) was restored to cells treated with CPA (100 \(\mu\)M, 15 min) in nominally Ca\(^{2+}\)-free HBS. The resulting SOCE was unaffected by bafilomycin A\(_1\) (1 \(\mu\)M, 1 h). Results show means ± s.e.m. from triplicate determinations within a single experiment. (D) SOCE after restoration of different concentrations of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_e\)) to control and bafilomycin A\(_1\)-treated cells (1 \(\mu\)M, 1 h). Results are means ± SEM from 3 independent experiments. These results confirm that when CPA (rather than thapsigargin, Fig. 5) is used to deplete the ER of Ca\(^{2+}\), bafilomycin A\(_1\) potentiates the Ca\(^{2+}\) signals evoked by Ca\(^{2+}\) release from the ER without affecting SOCE.
Fig. S8. Reducing luminal free \([\text{Ca}^{2+}]\) within the ER using a \([\text{Ca}^{2+}]\) buffer evokes SOCE that is insensitive to bafilomycin A1. (A) HEK cells were incubated with TPEN (100 \(\mu\text{M}, 2\ \text{min}\)) in nominally \([\text{Ca}^{2+}]\)-free HBS to reduce the luminal free \([\text{Ca}^{2+}]\) of the ER. Traces show the effects of then restoring extracellular \([\text{Ca}^{2+}]\) (HBS with 30 mM CaCl\(_2\)) to cells with or without bafilomycin treatment (Baf A\(_1\), 1 \(\mu\text{M}, 1\ \text{h}\)). (B) Experiments similar to those shown in A, but without TPEN. The results establish that TPEN evokes SOCE. (C) HEK cells loaded with TPEN as shown in A were stimulated with CCh (1 mM) in nominally \([\text{Ca}^{2+}]\)-free HBS with or without pre-treatment with bafilomycin A1 (1 \(\mu\text{M}, 1\ \text{h}\)). Traces show means ± s.e.m. (C, \(n=3\)) or ± range (A,B, \(n=2\)) from a single experiment, typical of at least 3 similar experiments. These results demonstrate that SOCE evoked by reducing luminal free \([\text{Ca}^{2+}]\) in the ER without \([\text{Ca}^{2+}]\) release is insensitive to bafilomycin A1, while the \([\text{Ca}^{2+}]\) signals resulting from CCh-evoked \([\text{Ca}^{2+}]\) release are potentiated.
Fig. S9. Colocalisation of ER markers in COS-7 cells. TIRFM images of COS-7 cells co-transfected with SERCA-mCherry and either GFP-ER, GFP-Ins(1,4,5)P$_3$ (IP$_3$)R1 or GFP-Ins(1,4,5)P$_3$ (IP$_3$)R3. Scale bar applies to all images. The colocalisations were statistically significant ($P<0.05$) with R$_{coloc}$ of 0.86±0.04 (SERCA-mCherry with GFP-Ins(1,4,5)P$_3$R1), 0.87±0.03 (SERCA-mCherry with GFP-Ins(1,4,5)P$_3$R3) and 0.79±0.06 (SERCA-mCherry with GFP-ER) (n=3 cells for each condition).
**Movie 1. Lysosomes remain associated with ER as they move.** TIRFM video showing a live COS-7 cell co-transfected with GFP-ER (green) and LAMP1-mCherry (red). Images were acquired every 322 ms and every third image is included in the video (i.e. a frame every 966 ms). The video is shown at 12 frames per second (i.e. video is running approximately 11.6× faster than real time). The field corresponds to that shown in Fig. 7.
Table S1. Disruption of lysosomes potentiates CCh-evoked Ca^{2+} signals in HEK cells

<table>
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<tr>
<th>Cell populations</th>
<th>Basal</th>
<th>Peak</th>
<th>pEC_{50}</th>
<th>Single cells</th>
<th>Basal</th>
<th>Peak</th>
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<tr>
<td></td>
<td>[Ca^{2+}]_i</td>
<td>%</td>
<td></td>
<td>[Ca^{2+}]_i</td>
<td>%</td>
<td>cells</td>
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<td></td>
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<td></td>
<td>M</td>
<td>nM</td>
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<tr>
<td>Control</td>
<td>25 ± 1</td>
<td>100</td>
<td>4.3 ± 0.2</td>
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<td>Bafilomycin A_{1}</td>
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<td>187 ± 28</td>
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<tr>
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<td>100</td>
<td>4.7 ± 0.1</td>
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<td>100</td>
<td>93 ± 1</td>
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<tr>
<td>Vacuolin</td>
<td>30 ± 2</td>
<td>145 ± 12</td>
<td>4.9 ± 0.1</td>
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<td>196 ± 22</td>
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<tr>
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<td>146 ± 14</td>
<td>4.7 ± 0.01</td>
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Effect of bafilomycin A_{1} (1 µM, 1 h), vacuolin (10 µM, 1 h) or GPN (200 µM, 30 min) on basal [Ca^{2+}]_i and peak Ca^{2+} signals evoked by CCh (1 mM) in population (n ≥ 3) and single-cell assays (n = 4 slides, with ~50 cells analysed on each) of HEK cells.