Calmidazolium and arachidonate activate a calcium entry pathway that is distinct from store-operated calcium influx in HeLa cells

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Agonists that deplete intracellular Ca^{2+} stores also activate Ca^{2+} entry, although the mechanism by which store release and Ca^{2+} influx are linked is unclear. A potential mechanism involves 'store-operated channels' that respond to depletion of the intracellular Ca^{2+} pool. Although SOCE (store-operated Ca^{2+} entry) has been considered to be the principal route for Ca^{2+} entry during hormonal stimulation of non-electrically excitable cells, recent evidence has suggested that alternative pathways activated by metabolites such as arachidonic acid are responsible for physiological Ca^{2+} influx. It is not clear whether such messenger-activated pathways exist in all cells, whether they are truly distinct from SOCE and which metabolites are involved. In the present study, we demonstrate that HeLa cells express two pharmacologically and mechanistically distinct Ca^{2+} entry pathways. One is the ubiquitous SOCE route and the other is an arachidonate-sensitive non-SOCE. We show that both these Ca^{2+} entry pathways can provide long-lasting Ca^{2+} elevations, but that the channels are not the same, based on their differential sensitivity to 2-aminoethoxydiphenyl borate, LOE-908 {(*R*,*S*)-(3,4-dihydro-6,7-dimethoxy-isochinolin-1-yl)-2-phenyl-*N*,*N*-di[2-(2,3,4-trimethoxyphenyl)ethyl]acetamid mesylate} and gadolinium. In addition, non-SOCE and not SOCE was permeable to strontium. Furthermore, unlike SOCE, the non-SOCE pathway did not require store depletion and was not sensitive to displacement of the endoplasmic reticulum from the plasma membrane using jasplakinolide or ionomycin pretreatment. These pathways did not conduct Ca^{2+} simultaneously due to the dominant effect of arachidonate, which rapidly curtails SOCE and promotes Ca^{2+} influx via non-SOCE. Although non-SOCE could be activated by exogenous application of arachidonate, the most robust method for stimulation of this pathway was application of the widely used calmodulin antagonist calmidazolium, due to its ability to activate phospholipase A_2.

Key words: arachidonic acid, calcium, calmidazolium, calmodulin, inositol, phospholipase.

INTRODUCTION

Calcium (Ca^{2+}) is a universal and versatile intracellular messenger that regulates a diverse range of cellular processes [1]. Cells have access to two sources of calcium, finite stores located in intracellular organelles and a more substantial pool of extracellular Ca^{2+}. In many cells types, release of Ca^{2+} from intracellular stores leads to the activation of a Ca^{2+} influx pathway denoted as SOCE (store-operated Ca^{2+} entry) [2]. This mechanism for promoting Ca^{2+} entry is responsible for replenishing depleted intracellular stores and prolonging cellular Ca^{2+} signals [3]. The molecular identity of the channels responsible for SOCE and their precise mechanism of activation are unclear. The probable candidates are members of the TRPC (canonical transient receptor potential) family [3,4]. Several studies support this hypothesis and have shown that the expression or ablation of various TRPC isoforms can modulate SOCE responses (see e.g. [5–7]).

A prominent model for the stimulation of TRPs during SOCE activation proposes a direct interaction between these plasma membrane channels and Ca^{2+} release channels on the intracellular stores. The latter would serve to sense a decrease in luminal Ca^{2+} concentration, and via a conformational change transmit this information to promote SOCE channel opening [2,3,8,9]. A direct interaction between TRPs and two families of intracellular Ca^{2+} release channel, Ins(1,4,5)P_3Rs (inositol 1,4,5-trisphosphate receptors) and RyRs (ryanodine receptors) has been demonstrated [10,11] (see [3,12] for reviews). When the Ca^{2+} concentration on the luminal domain of Ins(1,4,5)P_3Rs or RyRs is sufficiently low, this interaction can lead to TRP channel activation. Interventions that prevent the coupling of intracellular channels with TRPs, using either peptides that prevent the interaction (see e.g. [13]) or formation of a subplasmalemmal actin barrier [14,15], inhibit SOCE activation.

SOCE can be readily demonstrated in almost all non-electrically excitable cells and in some excitable cells after hormonal/neurotransmitter stimulation or depletion of intracellular Ca^{2+} stores using SERCA (sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase) inhibitors, such as thapsigargin [2,3,9,12]. The broad expression of the SOCE pathway has led to it being considered a prominent mechanism for Ca^{2+} entry into cells after Ca^{2+} pool discharge. However, it is becoming increasingly apparent that stimulation of cells with hormones that invoke the production of Ca^{2+}-releasing messengers not only activates SOCE, but can also promote additional Ca^{2+} entry pathways [16,17].

When Ins(1,4,5)P_3 is produced after phosphoinositide hydrolysis, there is a concomitant production of DAG (diacylglycerol).

Abbreviations used: AA, arachidonic acid; ACA, *N*-(*p*-amylcinnamoyl) anthranillic acid; 2-APB, 2-aminoethoxydiphenyl borate; CaM, calmodulin; DAG, diacylglycerol; GFP, green fluorescent protein; EGFP, enhanced GFP; EM, extracellular medium; ER, endoplasmic reticulum; Ins(1,4,5)P_3R, inositol 1,4,5-trisphosphate receptor; LOE-908, (*R*,*S*)-(3,4-dihydro-6,7-dimethoxy-isochinolin-1-yl)-2-phenyl-*N*,*N*-di[2-(2,3,4-trimethoxyphenyl)ethyl]acetamid mesylate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; RyR, ryanodine receptor; SOCE, store-operated Ca^{2+} entry; TFP, trifluoperazine; TRPC, canonical transient receptor potential; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide.

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Unlike water-soluble Ins(1,4,5)P_3, DAG stays in the plane of the plasma membrane where it can activate PKC (protein kinase C) or is metabolized further. Both PKC and DAG (or membrane-permeant analogues) have been demonstrated to cause Ca^{2+} influx distinct from SOCE in some cell types [18,19]. Furthermore, other messengers resulting from DAG metabolism, including AA (arachidonic acid) and leukotrienes, activate non-SOCE Ca^{2+} influx [20–22]. The molecular target of these messengers is not established, although TRPC isoforms have again been implicated. Although there are an increasing number of reports demonstrating significant Ca^{2+} influx via non-SOCE, at present, it is not clear whether all cells employ a non-SOCE mechanism, and if so, in response to which messenger. Probably, the best-characterized non-SOCE activator is AA. Studies from both the Shuttleworth [21] and Taylor [20] labs have demonstrated pathways for AA-stimulated Ca^{2+} entry that are clearly distinct from SOCE.

CaM (calmodulin) plays a critical role in transducing the effects of cytosolic Ca^{2+} signals on cellular processes. It is also involved in the regulation of many channels that generate Ca^{2+} signals. For example, two of the most prominent mechanisms for releasing intracellular Ca^{2+} stores, via Ins(1,4,5)P_3Rs and RyRs, have been demonstrated to be regulated by CaM [23,24]. For Ins(1,4,5)P_3Rs this is a largely negative effect, whereas for RyRs CaM can mediate both inhibition and activation (see [25,26] for reviews). CaM has also been demonstrated to modulate negatively Ca^{2+} influx through SOCE and TRP channels [27–30].

A common approach in investigating the action of CaM has been to use membrane-permeant pharmacological antagonists such as calmidazolium, W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide] and TFP (trifluoperazine). However, these agents have been shown to cause robust Ca^{2+} signals albeit through unknown mechanisms. Calmidazolium, for example, has been shown to cause Ca^{2+} release and Ca^{2+} entry in *Dictyostelium* [31], platelets [32], thyroid FRTL-5 cells [33], Madin–Darby canine kidney cells [34] and HL-60 cells [35]. These responses could be consistent with CaM causing a tonic inhibition of Ca^{2+} channels, which is relieved during activation or application of CaM antagonists.

We investigated the mechanism by which agents such as calmidazolium triggered Ca^{2+} increases using HeLa cells. Calmidazolium was capable of evoking Ca^{2+} signals that fully mimicked those stimulated by Ins(1,4,5)P_3-generating agonists in all respects except for the pathway through which Ca^{2+} influx occurred, i.e. via non-SOCE and not via SOCE.

MATERIALS AND METHODS

HeLa cell culture

HeLa cell culture was performed as described previously [36]. All experimental procedures were performed at room temperature (20–22 °C). Before imaging, the culture medium was replaced with an EM (extracellular medium) containing (mM): NaCl, 121; KCl, 5.4; $MgCl_2$, 0.8; $CaCl_2$, 1.8; $NaHCO_3$, 6; D-glucose, 5.5; Hepes, 25 (pH 7.3). Histamine, calmidazolium, U73122 and thapsigargin were obtained from Sigma. Fura 2 was obtained from Molecular Probes (Eugene, OR, U.S.A.). Statistics were performed using Student's *t* test (GraphPad Prism, San Diego, CA, U.S.A.).

Video imaging

Measurement of cytosolic Ca^{2+} in HeLa cells was performed by monitoring fura 2 fluorescence of cells adhered to glass coverslips using either a Spex or a PerkinElmer imaging system. Fura 2 was loaded into the cells by incubation with 2 μM fura 2 acetoxymethyl ester (30 min incubation followed by a 30 min period for de-esterification). For the Spex system, cells on coverslips were mounted on a Nikon Diaphot inverted epi-fluorescence microscope. Fluorescent images were obtained by alternate 40 ms excitations at 340 and 380 nm using twin xenon arc lamps each coupled with a spex monochrometer (Spex Industries, Edison, NJ, U.S.A.), with the wavelengths being switched by a rotating chopper mirror (Glen Creston Instruments, Stanmore, U.K.). Emission signals at 510 nm were collected using an intensified charge-coupled device video camera (Photonics Science, Robertson, U.K.) and filtered with a 200 ms time constant (Spex system) before off-line storage for analysis using an Imagine image processing system (Synoptics, Cambridge, U.K.).

With the PerkinElmer system, a single glass coverslip with adherent cells was mounted on the stage of a Nikon Diaphot 300 inverted epi-fluorescence microscope coupled with a xenon arc lamp (Nikon, Tokyo, Japan) light source. Fluorescence images were obtained with alternate excitations at 340 and 380 nm, selected for using either a Sutter filter wheel (340HT15 and 380HT15; Sutter Industries, Novarta, CA, U.S.A.) or a Spectramaster II monochrometer. Emitted light was filtered at 510 nm and collected by a cooled Astrocam digital camera. The acquired images were stored and subsequently processed off-line with Ultraview software (PerkinElmer LifeSciences, Great Shelford, Cambridge, U.K.).

Mn^{2+} entry was measured indirectly by recording the quench of fura 2 fluorescence when excited at 360 nm. To minimize the effect of contaminating Ca^{2+}, Mn^{2+}-containing Hepes buffer was supplemented with 1 mM EGTA and the Mn^{2+} concentration was adjusted to avoid chelation (2 mM Mn^{2+} and free Mn^{2+} $\sim$ 1 mM). MaxChelator (http://www.stanford.edu/$\sim$cpatton/maxc.html) was used to match free and total chelator and metals in all solutions containing Mn^{2+}, Gd^{3+} and Sr^{2+}.

Results are expressed as means $\pm$ S.E.M. Representative traces are shown for most of the experiments. These depict the most consistent pattern of response from multiple cells imaged in at least three independent experiments, with experiments repeated on different days. Statistical significance was calculated using Student's *t* test.

Expression of type 1 Ins(1,4,5)P_3 5′-phosphatase

The cDNA encoding the type 1 Ins(1,4,5)P_3 5′-phosphatase was amplified by PCR from a cDNA clone kindly provided by Professor C. Erneux (Université Libre de Bruxelles, Brussels, Belgium) and subcloned into pdc515-EGFP-C1 (where EGFP stands for enhanced green fluorescent protein). All constructs were transiently transfected using GeneJuice™ (Novagen, Nottingham, U.K.), according to the manufacturer's instructions. Briefly, 24 h before transfection, cells were seeded on to 22 mm glass coverslips at 50–80 % confluency. Cells were incubated for 24 h post-transfection at 37 °C, in 5 % CO_2 atmosphere with saturated humidity to allow expression of the construct. GFP-tagged actin was transfected into cells using the same procedure.

Jasplakinolide treatment

To stimulate cortical actin polymerization, cells were incubated for 30 min at 22 °C, with 10 μM jasplakinolide in nominally Ca^{2+} free solution. To confirm the effect of jasplakinolide on actin rearrangement, images of cells expressing GFP-tagged actin were acquired using a Bio-Rad MRC1024 confocal laser-scanning microscope ($\times$ 60 objective; 1.4 NA). GFP was excited using a 488 nm laser line and emission was collected through a 505 nm long-pass filter. Optical sections were taken every 0.4 μm.

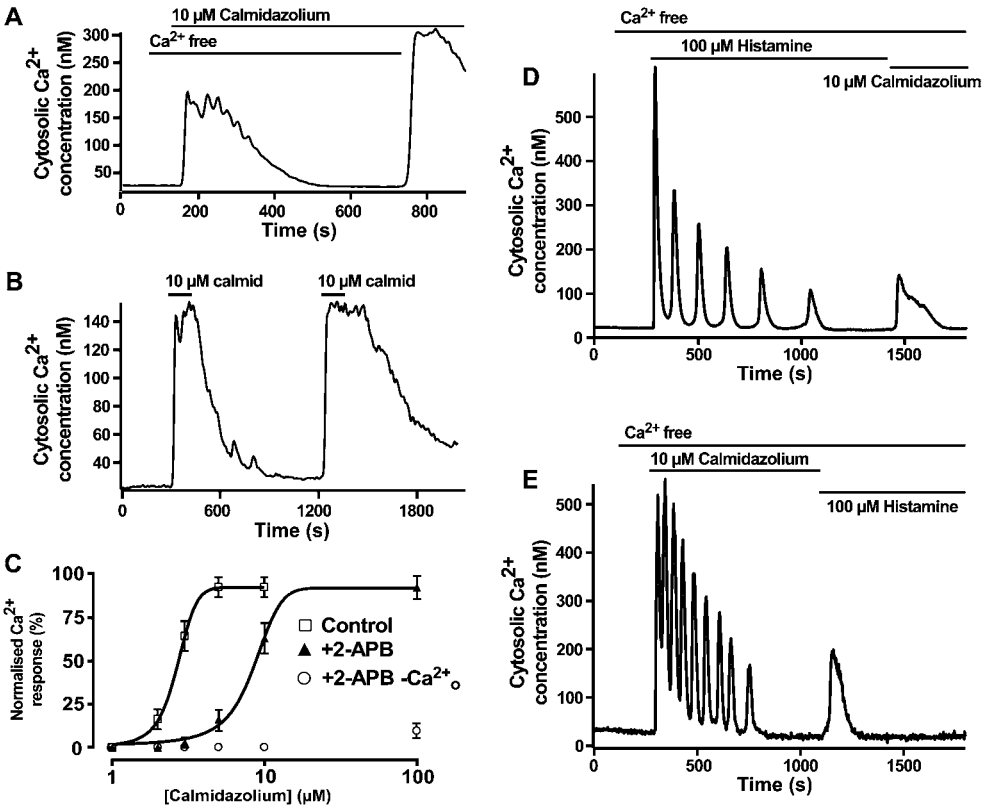

Figure 1 Characterization of calmidazolium-evoked Ca^{2+} transients

(**A**) Ca^{2+} mobilization and entry induced by calmidazolium in HeLa cells are depicted. Cells were perfused with 10 μM calmidazolium in a nominally Ca^{2+}-free medium before being perfused with 10 μM calmidazolium in the presence of extracellular Ca^{2+}. (**B**) Reversibility of cytosolic Ca^{2+} increase activated by a maximal calmidazolium concentration (2 min application followed by a 12 min continuous wash) is shown. The concentration–response relationship for calmidazolium-induced Ca^{2+} signalling is depicted in (**C**). To distinguish the concentration dependence for calmidazolium-mediated SOCE activation versus stimulation of the non-SOCE pathway, 100 μM 2-APB was added as shown in the key. In the presence of 2-APB and absence of extracellular Ca^{2+} (denoted $Ca^{2+}{}_o$ in the Figure), calmidazolium had a negligible effect on cellular Ca^{2+}. Results indicate means $\pm$ S.E.M. for three independent experiments with at least ten cells analysed per experiment for each condition. (**D**, **E**) Overlap between the histamine- and calmidazolium-releasable Ca^{2+} pool. The traces in (**A**, **B**, **D** and **E**) were obtained from single HeLa cells and are typical of at least 20 cells analysed in three independent experiments.

Image analysis and processing were performed with the public domain software ImageJ (NIH, http://rsb.info.nih.gov.ij).

Preparation and storage of AA

Porcine liver AA (Na^+ salt; Calbiochem) was dissolved in MilliQ water as a concentrated stock solution before being dispensed in aliquots; they were then frozen and stored at -20 °C in a light-resistant container. Before use, an aliquot of AA was diluted to the required concentration by addition of EM containing 0.1 % DMSO (to aid membrane permeability). All samples of AA were kept in the dark and on ice to suppress oxidation.

Use of Ba^{2+} as a Ca^{2+} surrogate

For some experiments, Ba^{2+} was used to monitor cation entry in place of Ca^{2+}. Ba^{2+} had been used in previous studies as a Ca^{2+} surrogate to provide a measure of unidirectional cation flux, since it was poorly sequestered by intracellular Ca^{2+} pumps or extruded from cells. Increases in cytosolic Ba^{2+} were monitored using fura 2 as described above, using the same excitation and emission settings as for Ca^{2+}. The results are presented as uncalibrated ratio (emission at 340 nm/excitation at 380 nm) units.

For the experiments shown in Figure 8, Ba^{2+} was employed simply because we could not use calcium with ionomycin-treated cells, since it would be impossible to identify Ca^{2+} that entered cells through the ionophore or via SOCE/non-SOCE. In our experiments, Ba^{2+} did not permeate into cells via ionomycin.

RESULTS

CaM antagonist calmidazolium evokes calcium release and activates a non-SOCE

CaM has been proposed to suppress the activation of both Ins(1,4,5)P_3Rs and SOCE channels. A common method of antagonizing the action of CaM is to use membrane-permeant pharmacological antagonists, such as calmidazolium, W-7 and TFP. Consistent with the idea that CaM antagonists relieve CaM-dependent inhibition of Ca^{2+} channels, these compounds have all been demonstrated to cause Ca^{2+} signals within intact cells. However, it has not been established whether their cellular target is CaM or other processes involved in Ca^{2+} signal transduction. We therefore determined whether there was a direct effect of CaM antagonists on the activity of Ins(1,4,5)P_3Rs and SOCE channels. We initially concentrated on the action of the imidazole compound calmidazolium, since this has been widely used to abrogate effects of CaM and has also been shown to activate Ca^{2+} mobilization and Ca^{2+} entry.

When applied in Ca^{2+}-free medium, calmidazolium evoked a rapid transient increase in cytosolic Ca^{2+} (Figure 1A; average

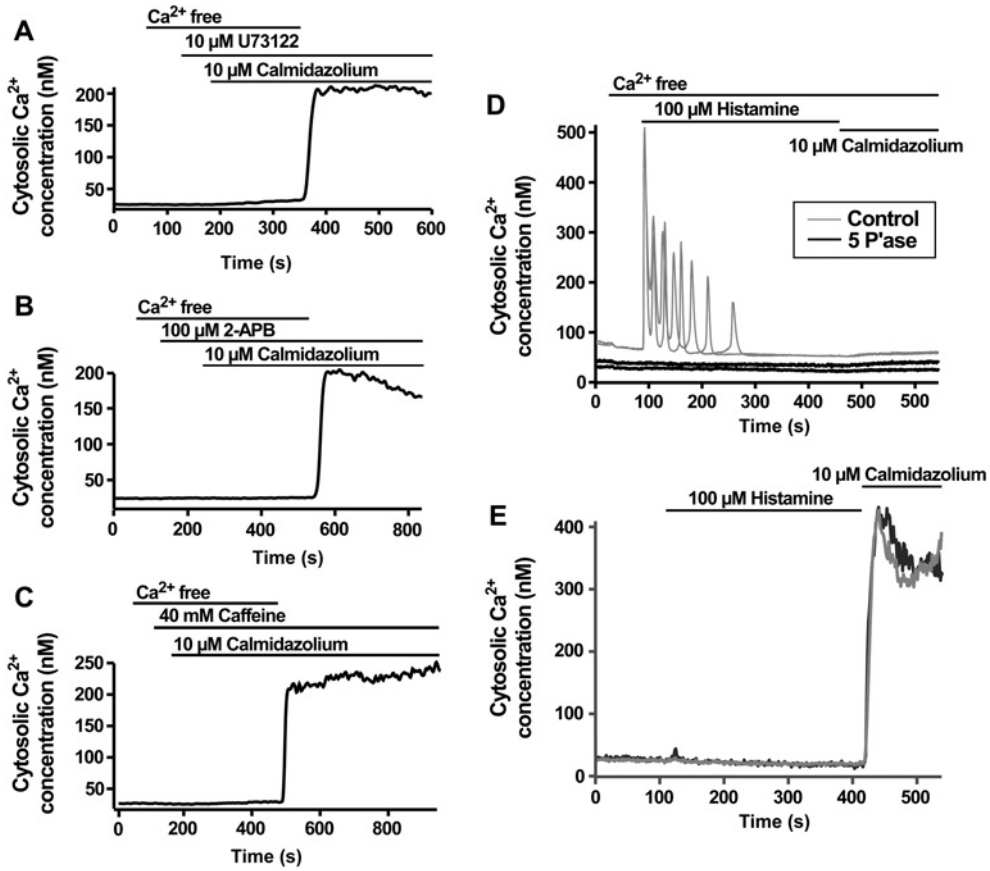

Figure 2 Activation of Ca^{2+} entry by calmidazolium does not require Ca^{2+} store release

(**A**–**C**) Lack of Ca^{2+} release after calmidazolium application if Ins(1,4,5)P_3 production or Ins(1,4,5)P_3Rs are inhibited using pharmacological agents. (**D**, **E**) A similar inhibition of calmidazolium-induced Ca^{2+} release was observed in cells overexpressing the Ins(1,4,5)P_3 metabolizing 5′-phosphatase enzyme (denoted 5 P′ase in the Figure). Note that the control traces in (**D**) have been shifted upwards by 50 nM to allow clearer visualization of the responding control and non-responding test traces. There was no difference in the basal Ca^{2+} levels between control and 5′-phosphatase-expressing cells. Representative traces from two individual cells are shown for both the control and 5′-phosphatase-expressing cells. Although overexpression of the 5′-phosphatase enzyme blocks calmidazolium-induced Ca^{2+} release, the traces in (**E**) show that calmidazolium could still activate Ca^{2+} influx. The responses of two representative 5′-phosphatase-expressing cells are shown. Histamine was added during the experiment to confirm the abolition of Ins(1,4,5)P_3-induced Ca^{2+} signals by the 5′-phosphatase expression. The traces in (**A**–**C**) were obtained from single HeLa cells and are typical of at least 20 cells analysed in three independent experiments.

peak response 462 ± 44 nM; $n = 126$ cells), which was followed by robust calcium entry when extracellular calcium was replaced (average response 935 ± 71 nM; $n = 126$ cells). Effects of calmidazolium were reversible, since pulsatile application caused repetitive Ca^{2+} responses (Figure 1B). Calmidazolium evoked concentration-dependent Ca^{2+} increases (Figure 1C), with all cells responding to doses of calmidazolium $> 2\ \mu$M ($n > 600$ cells from 20 independent experiments). At concentrations $\leqslant 1\ \mu$M calmidazolium, global Ca^{2+} responses were not observed. Instead, subcellular Ca^{2+} transients that we, and others, have described as 'Ca^{2+} puffs' [37] were evoked (results not shown).

The Ca^{2+} pool mobilized by calmidazolium overlapped largely with the Ins(1,4,5)P_3-sensitive Ca^{2+} store. Application of a maximal histamine concentration (100 μM) in Ca^{2+}-free medium to deplete the Ins(1,4,5)P_3-sensitive Ca^{2+} pools decreased the response to a subsequent addition of calmidazolium (10 μM) usually to a single low-amplitude Ca^{2+} transient (Figure 1D). In the reciprocal experiment, application of a maximal calmidazolium concentration (10 μM) in Ca^{2+}-free medium substantially reduced the magnitude of response observed with an ensuing histamine challenge (Figure 1E). When applied to naive cells, 100 μM histamine evoked an average peak Ca^{2+} increase in 763 ± 12 nM ($n = 49$ cells). After calmidazolium treatment, histamine responses were reduced to 283 ± 16 nM ($n = 37$ cells). With cells in Ca^{2+}-free medium, but not Ca^{2+}-containing medium, calmidazolium frequently triggered Ca^{2+} oscillations that were similar in rise time and duration to those observed during hormonal stimulation (cf. Figures 1D and 1E).

Calmidazolium-activated Ca^{2+} signals described above were comparable in almost all respects with typical responses triggered by hormonal stimulation of HeLa cells [38]. Similar to hormonal agonists, it appeared that calmidazolium was capable of releasing the Ins(1,4,5)P_3-sensitive Ca^{2+} store and triggering Ca^{2+} entry. To determine the mechanism by which calmidazolium could activate Ca^{2+} release, we examined the effects of well-known inhibitors of PLC (phospholipase C) and Ins(1,4,5)P_3Rs. Application of the PLC inhibitor U73122 at a concentration that blocks agonist-evoked Ca^{2+} signalling in HeLa cells [39] completely inhibited Ca^{2+} mobilization by calmidazolium (Figure 2A). Whereas all cells responded to 10 μM calmidazolium under control conditions (see above), no cells displayed Ca^{2+} mobilization in response to 10 μM calmidazolium in the presence of U73122. The inactive analogue U73343 did not prevent Ca^{2+} release activated by 10 μM calmidazolium ($n = 60$ cells; results not shown). 2-APB (2-aminoethoxydiphenyl borate), which we have demonstrated previously to inhibit Ins(1,4,5)P_3-induced Ca^{2+} release in HeLa cells [40], also prevented Ca^{2+} mobilization by calmidazolium (Figure 2B). Caffeine, which acts as a combined blocker of PLC

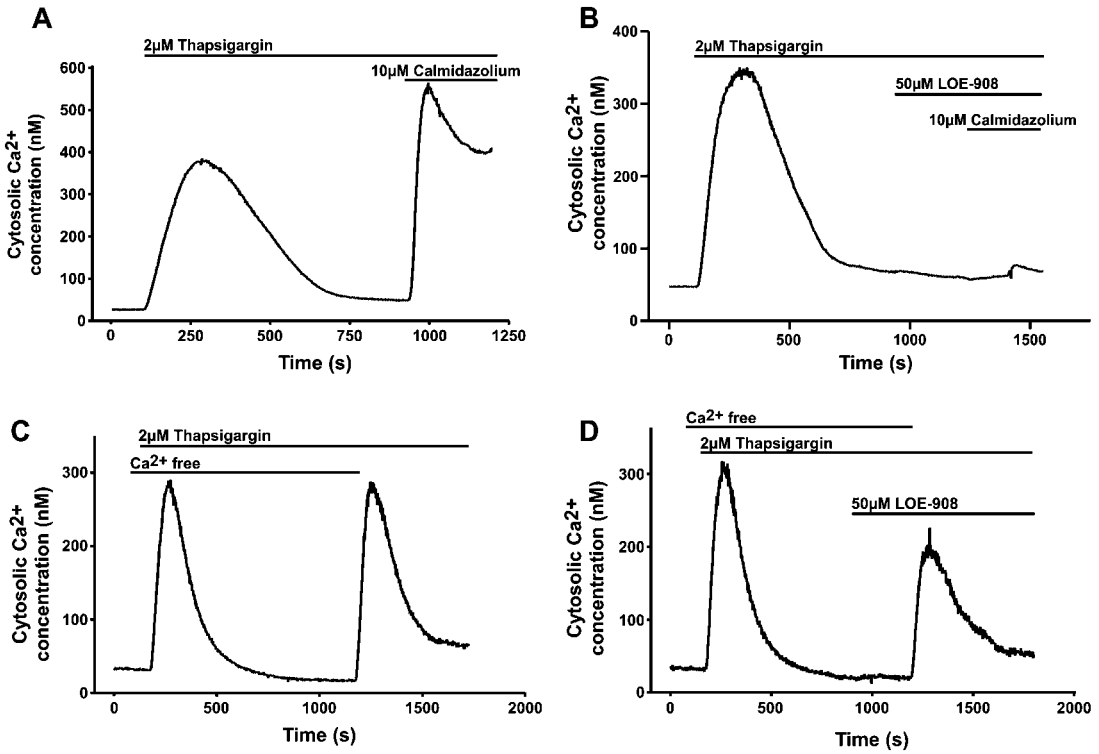

Figure 3 LOE-908 inhibits calmidazolium-induced Ca^{2+} entry, but not SOCE

(**A**–**D**) The responses of individual HeLa cells to the agents are denoted by the bars. For each panel, the response is typical of at least 20 cells analysed in three independent experiments.

and Ins(1,4,5)P_3Rs, also prevented calmidazolium-evoked Ca^{2+} release (Figure 2C). Heterologous expression of the 5′-phosphatase enzyme, which degrades Ins(1,4,5)P_3 to the non-Ca^{2+} releasing metabolite Ins(1,4)P_2, prevented Ca^{2+} mobilization in response to either a maximal histamine concentration (100 μM) or a maximal calmidazolium dose (10 μM; Figure 2D). Since calmidazolium has been shown previously to activate PLC activity [34], the simplest interpretation of the results presented above is that calmidazolium mobilizes Ca^{2+} stores by activation of PLC. Therefore, with respect to Ca^{2+} release from Ins(1,4,5)P_3Rs, calmidazolium mimics an Ins(1,4,5)P_3-generating agonist.

Although the mobilization of Ins(1,4,5)P_3-sensitive Ca^{2+} stores by calmidazolium can be ascribed to activation of PLC, the entry of Ca^{2+} in calmidazolium-treated cells appears to occur via a distinct mechanism. None of the agents that blocked calmidazolium-induced Ca^{2+} mobilization (Figures 2A–2C) inhibited Ca^{2+} entry when Ca^{2+} was reapplied to cells. In addition, expression of the 5′-phosphatase enzyme to levels that completely abrogated histamine-evoked Ca^{2+} signals and prevented calmidazolium causing Ca^{2+} release (Figure 2D), did not inhibit calmidazolium-induced Ca^{2+} entry (Figure 2E). Furthermore, similar to previous results [41], we have demonstrated that 2-APB completely inhibited SOCE in HeLa cells [40], yet it did not prevent calmidazolium-induced Ca^{2+} influx (Figure 2B). These results indicate that calmidazolium activated a Ca^{2+} entry pathway that was distinct from that triggered by store depletion.

Calmidazolium-activated non-SOCE is pharmacologically distinct from SOCE

Calmidazolium induced an additional increase in the plateau Ca^{2+} level after full SOCE activation by application of a maximal concentration of thapsigargin (2 μM; Figure 3A), which was completely blocked by the cation channel inhibitor LOE-908 {(*R*,*S*)-(3,4-dihydro-6,7-dimethoxy-isochinolin-1-yl)-2-phenyl-*N*,*N*-di[2-(2,3,4-trimethoxyphenyl)ethyl]acetamid mesylate}(Figure 3B). In contrast, LOE-908 gave only a modest inhibition of Ca^{2+} entry stimulated by thapsigargin (Figures 3C and 3D).

Gadolinium (Gd^{3+}) has been used previously as a high-affinity irreversible inhibitor of SOCE (see e.g. [42]). We similarly observed that 1 μM Gd^{3+} completely blocked Ca^{2+} influx evoked by application of thapsigargin (Figure 4A). However, Gd^{3+} had only a partial effect on calmidazolium-induced Ca^{2+} entry when applied at concentrations $\leqslant$ 10 μM (Figure 4B). The weak effect of Gd^{3+} on calmidazolium-evoked bivalent cation entry was also observed by substituting extracellular Ca^{2+} with manganese (Mn^{2+}) and by following the quenching of fura 2 fluorescence as Mn^{2+} entered the cells. Calmidazolium evoked a significant increase in the rate of Mn^{2+} entry (Figure 4C), which was unaffected by the presence of Gd^{3+} (Figure 4D). A further difference in cation sensitivity of the two influx pathways was observed when Ca^{2+} in the EM was substituted by strontium (Sr^{2+}). The SOCE pathway in HeLa cells did not appreciably conduct Sr^{2+}, whereas Sr^{2+} entry was readily detectable after calmidazolium treatment (Figure 4E). A summary of the pharmacological distinction between SOCE and the Ca^{2+} entry pathway activated by calmidazolium is shown in Figure 4(F).

During prolonged activation, SOCE-mediated Ca^{2+} influx diminishes despite intracellular Ca^{2+} stores remaining empty (see e.g. [43,44]; Figure 5A). This has been demonstrated to be due to progressive inhibition of the Ca^{2+} entry pathway and acceleration of Ca^{2+} sequestration [44]. Approx. 20 min after stimulation with thapsigargin, the cytosolic Ca^{2+} concentration had returned to the resting level. Subsequent application of calmidazolium provoked a steep increase in Ca^{2+} (Figure 5A), which was

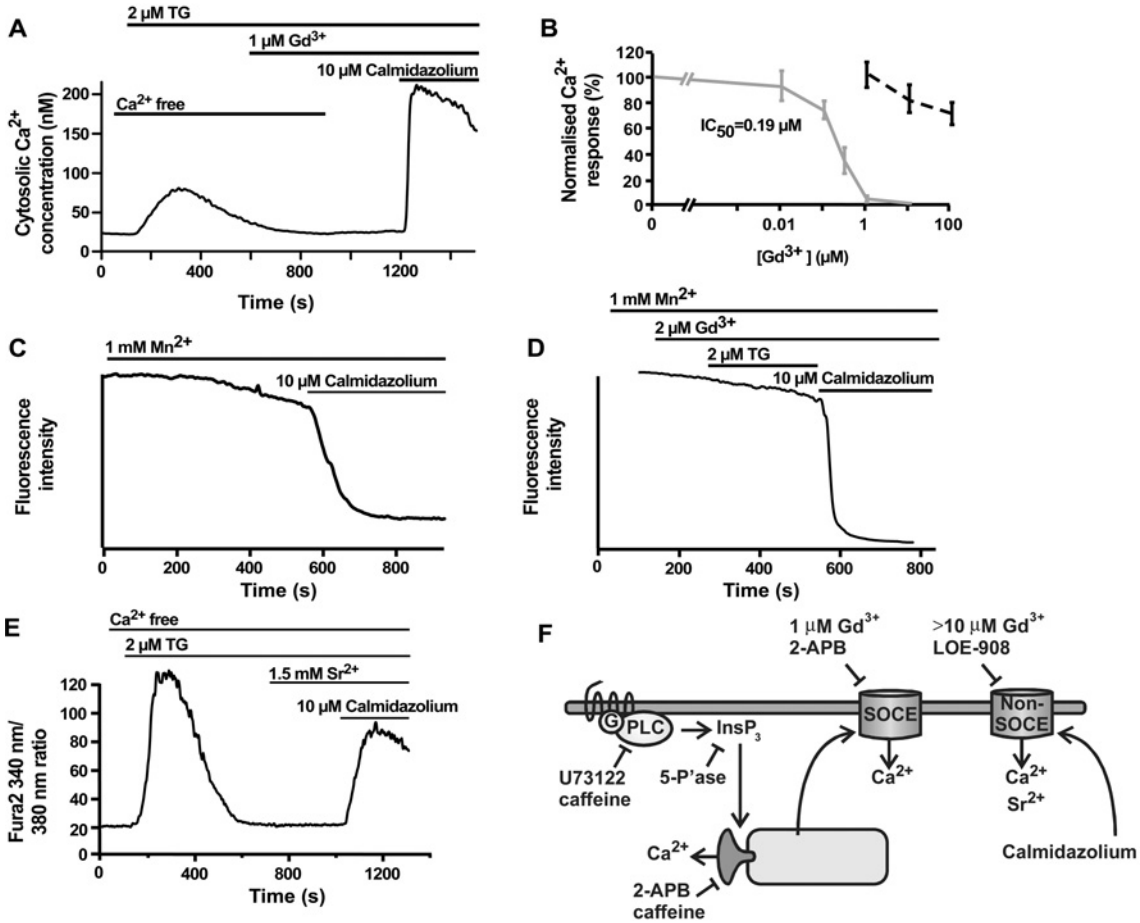

Figure 4 Characteristics of calmidazolium-activated non-SOCE

(**A**) Complete inhibitory effect of 1 μM Gd^{3+} on SOCE, with little effect on calmidazolium-evoked non-SOCE is illustrated. The concentration–response relationship for the effect of Gd^{3+} on SOCE and non-SOCE-mediated Ca^{2+} entry is shown in (**B**). The solid line depicts the effect of Gd^{3+} on thapsigargin (TG)-evoked SOCE, whereas the broken line illustrates the corresponding effect on calmidazolium-activated non-SOCE. (**C**, **D**) Calmidazolium activated entry of Mn^{2+} into HeLa cells, despite the presence of Gd^{3+} at a concentration that completely inhibits SOCE. (**E**) SOCE pathway in HeLa cells is not permeable to Sr^{2+}, but entry of this cation can be achieved by application of calmidazolium. A summary of the pharmacological profiles of the SOCE and non-SOCE cation entry pathways is depicted in (**F**). The traces shown in this Figure were obtained from individual HeLa cells and are indicative of similar responses in at least 20 cells from three independent experiments.

abolished by LOE-908 (results not shown). By itself, LOE-908 had little effect on the time course of thapsigargin-evoked SOCE (results not shown). Although the amplitude of both SOCE- and calmidazolium-evoked Ca^{2+} entries varied between cells, the latter pathway generally produced larger Ca^{2+} signals, as illustrated in Figure 5(B). For the experiment depicted in Figure 5(B), the mean peak amplitudes for Ca^{2+} entry through SOCE- and calmidazolium-sensitive pathway were 117 ± 33 and 275 ± 36 nM respectively ($n = 30$; $P < 0.002$).

In a recent study, Bolotina and co-workers [45] utilized calmidazolium to activate SOCE in smooth-muscle cells. They demonstrated that low concentrations of calmidazolium specifically activated SOCE, as judged by complete abolition of Ca^{2+} influx with 2-APB. At higher concentrations of calmidazolium, they found that the Ca^{2+} entry was insensitive to 2-APB. We observed a similar concentration-dependent action of calmidazolium. 2-APB suppressed responses to low ($\leqslant 2.5$ μM), but not high ($\geqslant 5$ μM), calmidazolium concentrations (Figure 1C). Similar to Bolotina and co-workers, we interpret these results as indicating that calmidazolium has distinct effects at low and high concentrations. Low concentrations activate SOCE, whereas higher doses switch the Ca^{2+} entry to 2-APB-insensitive non-SOCE.

Bolotina and co-workers [45] suggested that low calmidazolium concentrations activated SOCE in the absence of calcium release. In contrast, using real-time confocal microscopy, we observed that Ca^{2+} release was the earliest response of the cells to calmidazolium. As described above, concentrations of $\leqslant 1$ μM calmidazolium triggered Ca^{2+} puffs, which can lead to Ca^{2+} waves [37]. Therefore the most sensitive response of cells to calmidazolium is Ca^{2+} release, not Ca^{2+} entry.

Calmidazolium activates the same non-SOCE pathway as AA in HeLa cells

Various messengers and metabolites have been shown to activate Ca^{2+} influx independently of Ca^{2+} store release, including PKC, DAG and AA. We therefore used established pharmacological agents to probe the contribution of these moieties in calmidazolium-activated Ca^{2+} entry.

Treatment of cells with the PKC activators PMA (1 μM; $n =$ 50 cells) or 1-oleoyl-2-acetyl-*sn*-glycerol (1 μM; $n = 90$ cells) neither caused Ca^{2+} increase nor prevented Ca^{2+} response to calmidazolium when perfused on to cells for up to 30 min. The DAG lipase inhibitor, RHC-80267, has been used in previous

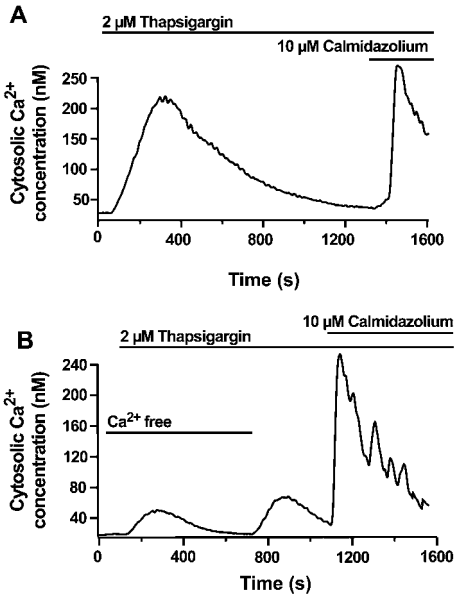

Figure 5 Calmidazolium can activate Ca^{2+} influx after run-down of SOCE

Prolonged activation of SOCE leads to a progressive decrease in Ca^{2+} to basal levels. (**A**) Application of calmidazolium activated Ca^{2+} entry despite run-down of SOCE is shown. (**B**) Method for estimating the relative amplitude of SOCE- and non-SOCE-mediated Ca^{2+} signals.

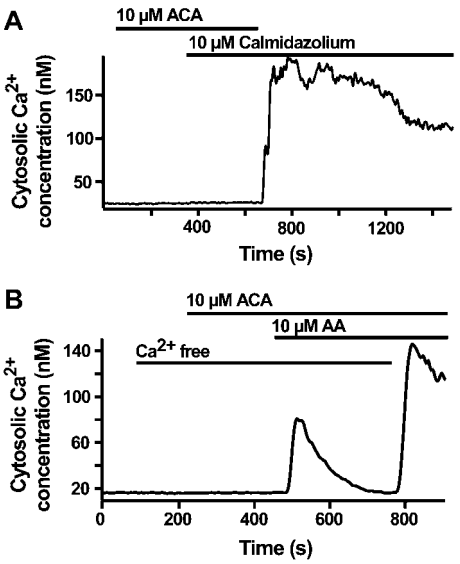

Figure 6 Calmidazolium-induced Ca^{2+} signalling is inhibited by the Ca^{2+}-independent PLA_2 inhibitor ACA

(**A**) The reversible inhibition of calmidazolium-evoked Ca^{2+} release and influx by ACA is depicted. (**B**) That AA could still release Ca^{2+} and activate non-SOCE in the presence of ACA, indicating that the PLA_2 inhibitor was not blocking non-SOCE channels is illustrated. The traces are taken from individual cells and are typical of at least 30 cells from three independent experiments.

studies (see e.g. [20]) to abrogate the release of AA from DAG and thus prevent the subsequent activation of an AA-sensitive Ca^{2+} influx. However, pretreatment of HeLa cells with RHC-80267 (50 μM; 15 min preincubation; $n = 25$ cells) did not affect the amplitude or extent of calmidazolium-induced Ca^{2+} entry.

Mammalian cells express a diverse array of PLA_2 (phospholipase A_2) subtypes [46]. In the present study, three PLA_2 inhibitors, ACA [*N*-(*p*-amylcinnamoyl) anthranillic acid], isotetrandrine and $AACOCF_3$ (arachidonyltrifluoromethyl ketone), were used to investigate the role of PLA_2 in the activation of Ca^{2+} entry by calmidazolium in HeLa cells. Isotetrandrine, an inhibitor of G-protein-linked PLA_2 (10 μM; 20 min preincubation; [21]) had no effect on calmidazolium-induced Ca^{2+} signals. Similarly, $AACOCF_3$, a selective inhibitor of cytosolic (85 kDa) PLA_2 (100 μM; 20 min preincubation) did not affect Ca^{2+} signals evoked by calmidazolium. In contrast, 10 μM ACA, which blocks Ca^{2+}-independent PLA_2 activity [47], rapidly and reversibly inhibited both Ca^{2+} mobilization and Ca^{2+} entry evoked by calmidazolium (Figure 6A). The ability of ACA to prevent calmidazolium-induced Ca^{2+} signals implicates AA or one of its metabolites as the critical messenger(s) underlying the activation of the non-SOCE Ca^{2+} influx pathway described above. Consistent with the notion that calmidazolium stimulates PLA_2 and that the resultant AA production activates Ca^{2+} signalling, direct application of 10 μM AA to cells in the presence of ACA evoked both Ca^{2+} mobilization and Ca^{2+} entry (Figure 6B).

To confirm that AA and calmidazolium were activating the same Ca^{2+} influx pathway, we examined the pharmacological profile and cation sensitivity of the Ca^{2+} signals evoked by direct AA application. Similar to calmidazolium, Ca^{2+} entry stimulated by 10 μM AA was not inhibited by 100 μM 2-APB (Figure 7A) or 10 μM U73122 (Figure 7B) or sensitive to expression of the 5′-phosphatase enzyme ($n = 10$ cells). Similar to the calmidazolium-provoked Ca^{2+} entry after run-down of SOCE (Figure 5A), AA evoked a substantial Ca^{2+} entry after the decrease in SOCE (Figure 7C), and this AA-induced Ca^{2+} entry was completely inhibited by LOE-908 (30 μM; $n = 35$ cells). The AA-activated pathway was also permeable to Sr^{2+} (Figure 7D) and not blocked by acute addition of 1 μM Gd^{3+} ($n = 120$ cells). In all respects, the Ca^{2+} influx pathway activated by AA resembled that observed with calmidazolium stimulation. Neither linolenic acid ($n = 60$ cells) nor linoleic acid ($n = 32$ cells) activated Ca^{2+} influx.

Although the experiments presented above indicate that AA production can stimulate the non-SOCE pathway, they do not implicate AA itself as the sole activator since it can be readily metabolized. We therefore examined the effect of inhibiting pathways responsible for AA metabolism. Lipoxygenase and cyclo-oxygenase enzymes convert AA into leukotrienes and prostaglandins, prostacyclins and thromboxanes respectively. Incubation of cells with the cyclo-oxygenase inhibitor indomethacin (10 μM; 25–90 min incubation in different experiments) did not alter basal Ca^{2+} levels or prevent Ca^{2+} signals evoked by calmidazolium ($n = 45$ cells). Similarly, the lipoxygenase inhibitor aspirin (100 μM; 30–90 min incubation in different experiments) had no effect ($n = 30$ cells). Finally, metabolism of AA via mono-oxygenases was prevented by using 100 μM metyrapone, which also did not alter Ca^{2+} mobilization or Ca^{2+} influx in response to calmidazolium ($n = 20$ cells). Calmidazolium belongs to a family of substituted imidazole compounds including econazole and miconazole. These compounds have a similar structure to calmidazolium, and may also affect CaM, although they are more commonly employed as inhibitors of cytochrome P450 mono-oxygenases. As with calmidazolium, 20 μM econazole and 20 μM miconazole evoked Ca^{2+} mobilization and Ca^{2+} entry. Co-application of maximal concentrations (10 μM calmidazolium, 20 μM econazole and 20 μM miconazole) of these imidazole compounds indicated that their Ca^{2+} responses were not additive (results not shown), suggesting that they activated the same Ca^{2+} influx pathway.

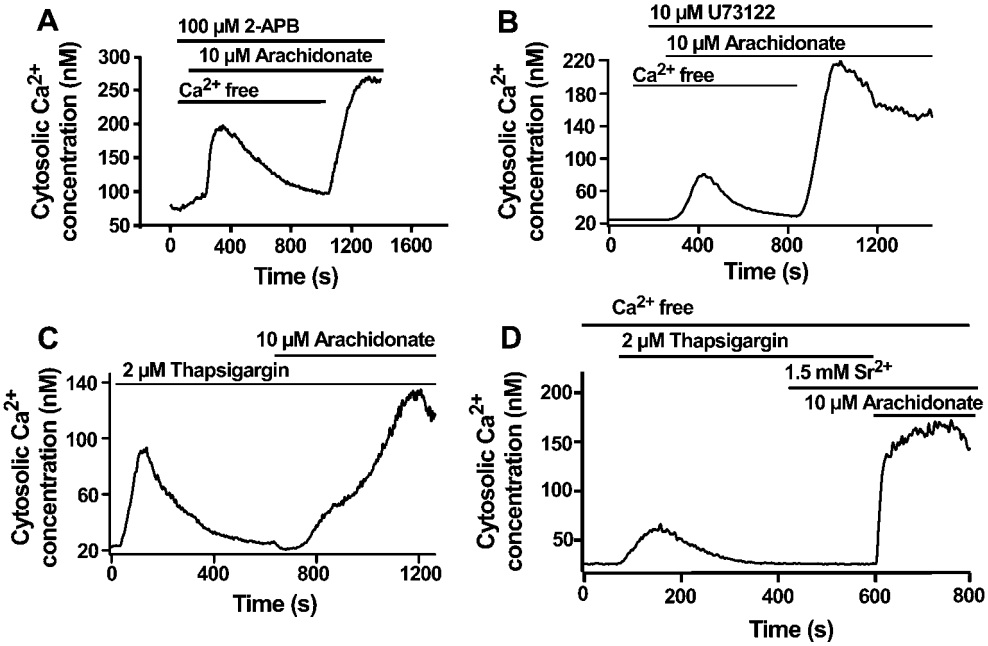

Figure 7 AA activates a non-SOCE pathway in HeLa cells

The panels in this Figure depict the action of AA in evoking Ca^{2+} influx with a similar pharmacology to calmidazolium-induced non-SOCE. The traces in (**A**–**D**) are taken from individual cells and are representative of at least 30 cells from three independent experiments for each panel.

Mechanistic differences between SOCE- and calmidazolium-activated non-SOCE

To distinguish further between SOCE and the AA-activated Ca^{2+} entry pathway in HeLa cells, we examined the effect of remodelling the cytoskeleton to produce a cortical actin deposition. This technique was used previously to inhibit SOCE activation [14,15,48]. Reorganization of the actin cytoskeleton was achieved by incubating HeLa cells with jasplakinolide (10 μM; 30 min preincubation), and monitored by confocal imaging of EGFP–actin-transfected cells. In control cells, there was a largely homogeneous pool of fluorescence, reflecting monomeric proteins, but with fluorescent stress fibres also visible. After treatment with jasplakinolide, the EGFP-tagged actin redistributed to the subplasmalemmal region of cells (Figure 8A). This treatment abolished the entry of barium (Ba^{2+}), which was used as a slowly permeant surrogate for Ca^{2+} through the SOCE pathway (Figure 8B). However, it did not prevent Ba^{2+} entry stimulated by calmidazolium (Figure 8B).

Similar to previous results [49], we have observed that incubation of cells with ionomycin causes retraction of the ER (endoplasmic reticulum) from the plasma membrane and fragmentation into discrete vesicles [50]. In Ca^{2+}-containing medium, such ionomycin-induced ER fragmentation and retraction occurs within approx. 5 min to 100% of cells examined ($n > 500$). Since the entry of Ca^{2+} itself cannot be measured in cells where ionomycin is present, we examined the effect of ER disruption on Ca^{2+} entry using Ba^{2+}. Despite the fact that ionomycin discharges the ER Ca^{2+} store and thus should activate SOCE, Ba^{2+} did not appear to permeate into ionomycin-treated cells by any detectable level. In contrast, addition of 10 μM calmidazolium stimulated Ba^{2+} entry significantly. These results indicate that the status and proximity of the ER to the plasma membrane can modulate Ca^{2+} entry through SOCE, but not the Ca^{2+} influx pathway activated by calmidazolium.

Effects of W-7 and TFP on Ca^{2+} signalling

We examined the effects of other structurally unrelated CaM antagonists to explore the possibility that activation of the non-SOCE pathway was somehow linked to their common action of CaM inhibition. In the absence of extracellular Ca^{2+}, both 300 μM W-7 and 10 μM TFP induced Ca^{2+} release from intracellular stores, which was followed by Ca^{2+} entry when extracellular Ca^{2+} was restored (results not shown). Unlike calmidazolium, W-7 was capable of releasing Ca^{2+} stores despite the presence of 100 μM 2-APB and activate Ca^{2+} entry in the presence of either 2-APB or LOE-908. TFP activated an LOE-908-sensitive Ca^{2+} influx, but unlike the substantial and robust Ca^{2+} influx signals evoked by calmidazolium, TFP triggered highly variable modest Ca^{2+} changes (results not shown). The distinct characteristics of the Ca^{2+} signals evoked by the CaM antagonists suggests that they have different modes of action, and that their ability to increase cytosolic Ca^{2+} is not due to their common ability to antagonize CaM.

DISCUSSION

Previous studies have demonstrated that application of CaM antagonists elicit Ca^{2+} signals, and proposed that this is due to relief of CaM-dependent Ca^{2+} channel inhibition. We similarly observed that calmidazolium evoked robust Ca^{2+} signals, but found that this action was due to stimulation of PLC and PLA_2. Calmidazolium-evoked responses in HeLa cells closely mimicked the profile of Ca^{2+} signals evoked by a PLC-coupled agonist (Figure 1). The release of Ca^{2+} from intracellular stores was blocked by U73122 and caffeine (Figure 2), established antagonists of PLC-mediated Ins(1,4,5)P_3 production. Furthermore, calmidazolium released Ca^{2+} from the same pool as histamine (Figures 1D and 1E), in an Ins(1,4,5)P_3-dependent manner (Figure 2D). Activation of PLC and production of Ins(1,4,5)P_3

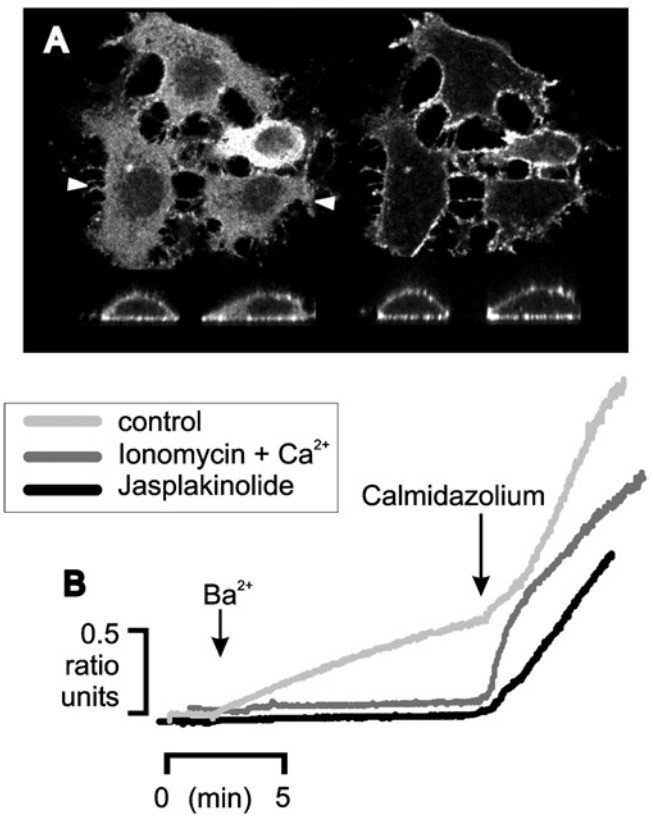

Figure 8 Cortical actin deposition or ionomycin-induced ER fragmentation blocks SOCE, but not calmidazolium-evoked non-SOCE

HeLa cells were treated with jasplakinolide as described in the Materials and methods section to induce the redistribution of actin from the cell centre to the periphery. The effect of jasplakinolide treatment is shown in (**A**). The left- and right-hand sides of the panel depict cells before and after jasplakinolide treatment respectively. Two-dimensional images and axial projections are shown to indicate the redistribution of actin. The axial projection was taken across the portion of the image between the white arrowheads. (**B**) Effect of pretreating cells with jasplakinolide or ionomycin on SOCE and their ineffectiveness on non-SOCE. After incubation with jasplakinolide or control buffer, the cells were stimulated with 1 μM thapsigargin in Ca^{2+}-free medium for 5 min to activate SOCE. For the ionomycin-treated cells, thapsigargin was not added since the ionophore by itself should have been sufficient to deplete the Ca^{2+} stores and activate SOCE.

therefore appears to underlie the release of intracellular Ca^{2+} stores by calmidazolium.

In addition to stimulation of PLC, calmidazolium activated a non-SOCE pathway. This Ca^{2+} entry mechanism was demonstrably different from SOCE based on its pharmacological profile (Figures 2A–2C, 3B and 4A–4D), ion selectivity (Figure 4E), lack of sensitivity to remodelling actin in a cortical ring (Figure 8B) or ionomycin-induced fragmentation and retraction of the ER (Figure 8B). The activation of non-SOCE by calmidazolium appeared to be due to stimulation of PLA_2 since inhibition of this enzyme completely prevented Ca^{2+} influx in response to calmidazolium. Furthermore, the product of PLA_2 activation, AA, was capable of stimulating a non-SOCE pathway with exactly the same pharmacological profile (Figures 7A and 7B) and ion selectivity (Figure 7D) as calmidazolium itself. Inhibition of lipoxygenase, cyclo-oxygenase and mono-oxygenase pathways did not alter the ability of calmidazolium to trigger Ca^{2+} influx, suggesting that AA is the messenger responsible for the activation of non-SOCE.

Of all the metabolites demonstrated to activate non-SOCE, possibly the most commonly effective moiety is AA. This polyunsaturated fatty acid has been demonstrated to activate Ca^{2+} influx in a variety of different cell types [7,20–22,51,52]. In the present study, we observed that addition of AA to cells rapidly inhibited SOCE and switched the mode of Ca^{2+} influx to LOE-908-sensitive non-SOCE (Figure 7C). Calmidazolium also switched the pathway of Ca^{2+} influx from SOCE to LOE-908-sensitive non-SOCE (Figure 3B), although it caused a more rapid changeover when compared with AA. These results suggest that AA is a dominant Ca^{2+} influx effector over the SOCE mechanism, and that the pathways do not function in an additive manner. We found that both calmidazolium and AA could activate Ca^{2+} entry despite an ongoing SOCE response (e.g. Figures 3A and 7C). Addition of either of the compounds at the peak or plateau phase of a thapsigargin response, triggered non-SOCE, suggesting that this mechanism is not particularly sensitive to ambient Ca^{2+} levels. It therefore appears to us that the exclusivity of these Ca^{2+} influx pathways reflects the dominance of AA, it shuts off SOCE and promotes non-SOCE.

Our results have some similarity to the recent study of Bolotina and co-workers [45], who demonstrated that low concentrations of calmidazolium stimulated 2-APB-sensitive SOCE, whereas higher doses of calmidazolium invoked a 2-APB-insensitive Ca^{2+} influx. Similar to the results of the present study, Bolotina and co-workers suggested that the key target of calmidazolium was PLA_2. They focused on the effects of low calmidazolium concentrations and suggested that the lysophospholipids produced by PLA_2 caused SOCE activation. We have characterized the Ca^{2+} entry generated by higher concentrations of calmidazolium and found that AA probably plays a significant role. Calmidazolium therefore appears to be capable of activating both SOCE and non-SOCE depending on its concentration. Both effects stem from the activation of PLA_2, but may depend on the balance between lysophospholipids and AA.

Although they are widely used, cellular actions of CaM antagonists are not fully resolved. The consistent observation that they increase cytosolic Ca^{2+} is problematic considering that they are generally used to antagonize downstream actions of Ca^{2+}. Concentration-dependent Ca^{2+} increases have been reported using various cell types in response to CaM antagonists including TFP, W-7, fendiline, chlorpromazine as well as calmidazolium. For most of these agents, the mechanism by which they cause Ca^{2+} signals is not known. Calmidazolium is perhaps one of the best understood and is known to affect Ins(1,4,5)P_3Rs [53], SERCA1 Ca^{2+} pumps [54] and L-type voltage-operated Ca^{2+} channels [55]. Consistent with our observations, calmidazolium has been demonstrated to cause Ins(1,4,5)P_3 production through activation of PLC in several cell types (see e.g. [34,56]). Furthermore, calmidazolium activated Ca^{2+} release and Ca^{2+} entry in Madin–Darby canine kidney cells, with the effects being abolished by the PLA_2 inhibitor aristolochic acid [34].

Accumulating evidence suggests that CaM is a significant regulator of SOCE activity. CaM may prevent Ca^{2+} entry by interfering with the physical coupling of Ins(1,4,5)P_3Rs and SOCE channels. C-terminal portions of different TRPCs have been demonstrated to bind CaM or an N-terminal sequence of Ins(1,4,5)P_3Rs in a mutually exclusive manner [27,57,58]. Displacement of CaM by short peptides corresponding to the competing region of Ins(1,4,5)P_3Rs leads to activation of TRP channels [30]. CaM therefore appears to act as a barrier in the activation of Ca^{2+} entry by occluding the site through which TRPs and Ins(1,4,5)P_3Rs interact. Consistent with this notion, application of CaM antagonists (W-7 and TFP) significantly reduced the delay in development of SOCE after store depletion, whereas increasing CaM concentration caused the converse [28]. Furthermore, calmidazolium was capable of enhancing TRP activity by displacing

CaM from the site where it prevented interaction with Ins(1,4,5)P_3Rs [30,57]. For Ins(1,4,5)P_3Rs, CaM can bind to an N-terminal region and decrease the ability of Ins(1,4,5)P_3 to activate the channels [24,59]. In addition, CaM may be responsible for the negative feedback effects of increased cytosolic Ca^{2+} on Ins(1,4,5)P_3R activation [23]. CaM therefore can provide both a tonic and a dynamic inhibitory influence on Ca^{2+} release and entry in non-electrically excitable cells.

Although we suppose that the ability of calmidazolium to release Ca^{2+} and activate non-SOCE was not due to CaM antagonism, we did observe an effect of calmidazolium on SOCE, which is consistent with the proposed inhibitory action of CaM. Activation of SOCE by thapsigargin allows the entry of Ca^{2+} or Ba^{2+} into HeLa cells. Ba^{2+} is a useful surrogate, since it is poorly transported by cellular Ca^{2+} pumps and therefore provides a unidirectional measure of cation entry. In the presence of ACA to prevent activation of non-SOCE, calmidazolium increased the rate of thapsigargin-stimulated Ba^{2+} entry into cells by approx. 7-fold. In the absence of calmidazolium, the initial rate of Ba^{2+} entry was 0.19 ± 0.04 ratio units/min ($n = 5$; measured using fura 2; see the Materials and methods section). In the presence of 10 μM calmidazolium, the rate of Ba^{2+} entry was 1.26 ± 0.21 ratio units/min ($n = 6$). It therefore appears that calmidazolium does potentiate SOCE by working as a conventional CaM antagonist.

In summary, we have demonstrated that HeLa cells express two distinct Ca^{2+} entry pathways, one regulated by store depletion and the other by AA. These pathways do not operate simultaneously due to the dominant effect of AA, which inhibits SOCE and promotes non-SOCE. The non-SOCE pathway can be directly activated by exogenous AA application, but in HeLa cells it is also robustly stimulated by calmidazolium. The effect of calmidazolium in promoting Ca^{2+} release and non-SOCE is not due to CaM antagonism, but rather due to activation of PLC and PLA_2. At present the function of the non-SOCE pathway in HeLa cells is not clear. Our attempts to find cellular agonists that couple with this pathway have so far proved negative. Histamine, ATP and serum all strongly activate Ca^{2+} release in HeLa cells, which is followed by Ca^{2+} entry. However, the Ca^{2+} influx was sensitive to 1 μM Gd^{3+}, which is indicative of SOCE. In addition, LOE-908 does not affect prolonged Ca^{2+} oscillations activated by histamine (results not shown). Calmidazolium can stimulate Ca^{2+} oscillations, but only in Ca^{2+}-free medium when the non-SOCE pathway is not transporting Ca^{2+} into the cells. Therefore, rather than supporting Ca^{2+} oscillations, the non-SOCE channel appears to compromise the ability of HeLa cells to display repetitive Ca^{2+} spikes.

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