Ca\textsuperscript{2+} Influx through Store-operated Calcium Channels Replenishes the Functional Phosphatidylinositol 4,5-Bisphosphate Pool Used by Cysteinyl Leukotriene Type I Receptors*

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Background: Phosphatidylinositol 4,5-bisphosphate levels need to be replenished during calcium signaling, but how this is achieved is unclear.

Results: Ca\textsuperscript{2+} entry through Orai1 channels contributes to the resynthesis of phosphatidylinositol 4,5-bisphosphate and, thereby, helps sustain cytoplasmic Ca\textsuperscript{2+} oscillations.

Conclusion: Ca\textsuperscript{2+} influx sustains cytoplasmic Ca\textsuperscript{2+} oscillations by regulating phosphatidylinositol-4-phosphate 5-kinase.

Significance: Store-operated Ca\textsuperscript{2+} influx is important in maintaining phosphatidylinositol 4,5-bisphosphate levels.

Oscillations in cytoplasmic Ca\textsuperscript{2+} concentration are a universal mode of signaling following physiological levels of stimulation with agonists that engage the phospholipid C pathway. Sustained cytoplasmic Ca\textsuperscript{2+} oscillations require replenishment of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}), the source of the Ca\textsuperscript{2+}-releasing second messenger inositol trisphosphate. Here we show that cytoplasmic Ca\textsuperscript{2+} oscillations induced by cysteinyl leukotriene type I receptor activation run down when cells are pretreated with Li\textsuperscript{+}, an inhibitor of inositol monophosphatases that prevents PIP\textsubscript{2} resynthesis. In Li\textsuperscript{+}-treated cells, cytoplasmic Ca\textsuperscript{2+} signals evoked by an agonist were rescued by addition of exogenous inositol or phosphatidylinositol 4-phosphate (PI4P). Knockdown of the phosphatidylinositol 4-phosphate 5 (PIP5) kinases \( \alpha \) and \( \gamma \) resulted in rapid loss of the intracellular Ca\textsuperscript{2+} oscillations and also prevented rescue by PI4P. Knockdown of talin1, a protein that helps regulate PIP5 kinases, accelerated rundown of cytoplasmic Ca\textsuperscript{2+} oscillations, and these could not be rescued by inositol or PI4P. In Li\textsuperscript{+}-treated cells, recovery of the cytoplasmic Ca\textsuperscript{2+} oscillations in the presence of inositol or PI4P was suppressed when Ca\textsuperscript{2+} influx through store-operated Ca\textsuperscript{2+} channels was inhibited. After rundown of the Ca\textsuperscript{2+} signals following leukotriene receptor activation, stimulation of P2Y receptors evoked prominent inositol trisphosphate-dependent Ca\textsuperscript{2+} release. Therefore, leukotriene and P2Y receptors utilize distinct membrane PIP\textsubscript{2} pools. Our findings show that store-operated Ca\textsuperscript{2+} entry is needed to sustain cytoplasmic Ca\textsuperscript{2+} signaling following leukotriene receptor activation both by refilling the Ca\textsuperscript{2+} stores and by helping to replenish the PIP\textsubscript{2} pool accessible to leukotriene receptors, ostensibly through control of PIP5 kinase activity.

A rise in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) is a universal signal used throughout the phylogenetic tree to activate a broad range of spatially and temporally distinct cellular responses ranging from exocytosis and fast-twitch muscle contraction to nuclear gene expression and cell proliferation (1, 2). Inherent to the use of a multifarious signal like Ca\textsuperscript{2+} is the problem of specificity. How can one Ca\textsuperscript{2+}-dependent response within a cell be activated but not another? Evidence is now accumulating that the size, time course, and subcellular location of the rise in [Ca\textsuperscript{2+}], are all important factors that contribute to the selective recruitment of downstream targets (2, 3).

In many cells types, stimulation of surface receptors that couple to heterotrimeric G\textsubscript{x} proteins and phospholipase C\textsubscript{β} and, thereby, hydrolyze the membrane phospholipid PIP\textsubscript{2} generate the second messenger InsP\textsubscript{3}, which releases stored Ca\textsuperscript{2+} by opening ligand-gated Ca\textsuperscript{2+} channels on the endoplasmic reticulum (ER) (4). Low levels of stimulation of these G-protein-coupled receptors, considered to represent physiological levels of receptor activation, often result in the generation of repetitive oscillations in [Ca\textsuperscript{2+}], that present either as a series of baseline Ca\textsuperscript{2+} spikes or slower sinusoidal Ca\textsuperscript{2+} waves on an elevated plateau (5). Information is encoded in the amplitude, frequency, and spatial profile of the oscillation (3). Oscillations in [Ca\textsuperscript{2+}], as opposed to a sustained rise in [Ca\textsuperscript{2+}], enhance mitochondrial energy production (6), exocytosis (7), and Ca\textsuperscript{2+}-dependent gene expression (8, 9) while avoiding the toxic effects that are associated with prolonged elevation of [Ca\textsuperscript{2+}],

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\footnote{2 The abbreviations used are: PIP\textsubscript{2}, phosphatidylinositol 4,5-bisphosphate; InsP\textsubscript{3}, inositol trisphosphate; ER, endoplasmic reticulum; IMPase, inositol monophosphatase; PI, phosphatidylinositol; PIPS, phosphatidylinositol 4-phosphate 5; PIP\textsubscript{2}, phosphatidylinositol 4-phosphate; LTC\textsubscript{4}, leukotriene C4; MJCD, methyl-β-cyclodextrin; CRAC, Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+}; STIM, stromal interaction molecule.}
Store-operated Ca\(^{2+}\) Entry Regulates PIP\(_2\) Levels

Oscillations in [Ca\(^{2+}\)]\(_i\) require either oscillations in the levels of InsP\(_3\) or biphasic gating of the InsP\(_3\) receptor by cytoplasmic Ca\(^{2+}\) in the presence of a steady increase in InsP\(_3\). Regardless of the mechanism, two conditions need to be satisfied for repetitive oscillations in [Ca\(^{2+}\)]\(_i\) to occur. First, the Ca\(^{2+}\) content of the stores must be maintained at a level sufficient for Ca\(^{2+}\) release. This is necessary because, following each Ca\(^{2+}\) release event, a fraction of the mobilized Ca\(^{2+}\) is extruded from the cell by plasma membrane Ca\(^{2+}\) ATPase pumps (10). In the absence of refilling, store Ca\(^{2+}\) content would therefore drop below the level that supports InsP\(_3\)-dependent Ca\(^{2+}\) release. Store refilling is accomplished through activation of store-operated CRAC channels in the plasma membrane that open following loss of Ca\(^{2+}\) from within the ER (11). CRAC channels are comprised of STIM and Orai proteins (12). STIM1 and STIM2 are ER Ca\(^{2+}\) sensors and migrate toward the plasma membrane upon store depletion (reviewed in Ref. 13). Orai1 is the pore-forming subunit of the CRAC channel and is gated by STIM binding to the C and N termini of the protein (reviewed in Ref. 14). The second criterion that needs to be satisfied for the generation of prolonged oscillations in [Ca\(^{2+}\)]\(_i\) is that PIP\(_2\) levels must be replenished following each Ca\(^{2+}\) spike to support production of the InsP\(_3\) that is needed for the next oscillation in [Ca\(^{2+}\)]\(_i\). InsP\(_3\) has a lifetime of just a few seconds in the cytoplasm (15, 16) because of the presence of strong catabolic pathways. These breakdown pathways are well characterized and involve sequential dephosphorylation by phosphatases to myoinositol monophosphate, which, in turn, is dephosphorylated to myoinositol monophosphate by inositol monophosphatases (IMPases), enzymes that are inhibited by Li\(^+\). Myoinositol combines with cytidine diphosphate diacylglycerol to form phosphatidylinositol (PI), which can then be phosphorylated by PI4 and PI5 kinases to make PIP\(_2\).

Two IMPase genes have been identified in mammals: IMPase 1 and IMPase 2. IMPase 2 is implicated in the pathogenesis of bipolar disorder, schizophrenia, and febrile seizures (17). Blocking of inositol monophosphatases, which would deplete PIP\(_2\) levels and, therefore, impair production of InsP\(_3\), is a possible mechanism to explain the mood-stabilizing effect of Li\(^+\) treatment on bipolar disorder (18). Although both IMPases are inhibited by Li\(^+\), the IMPase 1 is more sensitive to blocking, with an IC\(_{50}\) of 0.7 mM (in 2 mM MgCl\(_2\)) that is ~30-fold left-shifted compared with IMPase 2 inhibition (19).

PIP\(_2\) levels regulate CRAC channel activity in various ways. STIM 1 trafficking to the cell periphery is expedited by the presence of a cytoplasmic Lys-rich domain that may bind to PIP\(_2\) and phosphatidylinositol 3,4,5-trisphosphate in the inner leaflet of the plasma membrane (20, 21). Furthermore, slow Ca\(^{2+}\)-dependent inactivation of the channels requires Orai1 confinement to PIP\(_2\)-rich domains of the plasma membrane (22). Here we explored the possibility that Ca\(^{2+}\) influx through CRAC channels, in addition to refilling the stores, regulates replenishment of the agonist-sensitive PIP\(_2\) pool during oscillations in [Ca\(^{2+}\)]. We find that the step converting PI4P to PIP\(_2\) is dependent on Ca\(^{2+}\) entry through the channels, providing an auto-regulatory mechanism through which CRAC channels maintain their own activity by ensuring sufficient PIP\(_2\) levels in the plasma membrane for store depletion via InsP\(_3\) production.

Experimental Procedures

Reagents—Culture medium and salts were obtained from Sigma. LTC\(_4\) was purchased from Cambridge Bioscience Ltd. (Cambridge, UK). FCS, Fura-2/AM, Dulbecco’s modified Eagle’s medium, and penicillin-streptomycin were obtained from Invitrogen. Thapsigargin was purchased from Merck Chemicals Ltd. (Darmstadt, Germany). PI4P diC8 and unlabeled shuttle PIP carrier 3 were purchased from Echelon Biosciences Inc. Unlabeled shuttle PIP carrier 3 (catalog no. P-9C3) was used to deliver PI4P into cells. The carrier was reconstituted in distilled water and mixed with PI4P. The carrier-PI4P complex was vortexed and left at room temperature for 15–20 min. Following this, the complex was diluted in external solution and applied to intact cells for 7 min prior to stimulation with LTC\(_4\). The carrier final concentration was 20 μM.

Cell Culture—Rat basophilic leukemia RBL-2H3 cells were from the ATCC. Early experiments to see whether LTC\(_4\) evoked Ca\(^{2+}\) signals in this cell line were carried out on cells provided by Dr. Shamshad Cockcroft (University College London, London, UK). RBL-2H3 cells were used because of their strong secretory response, which is being investigated in a broader study within the group. Cells were routinely maintained in Dulbecco’s modified Eagle’s medium and supplemented with 10% FCS and 1% penicillin-streptomycin, as described previously (23). Cells were kept in an atmosphere containing 5% CO\(_2\) and maintained at 37 °C. For calcium imaging experiments, cells were plated onto 13-mm glass coverslips using 0.05% trypsin and used 48 h after passaging.

Cytoplasmic Ca\(^{2+}\) Measurements—A Nikon Eclipse TE2000-U inverted microscope equipped with an IMAGO camera-based system from TILL Photonics (Gräfelfingen, Germany) was used as described previously (23). Cells were alternately excited at 356/380 nm with exposure times of 20 ms and an acquisition rate of 0.5 Hz. Data were analyzed after import to IGOR Pro (Wave Metrics, Lake Oswego, OR). All experiments were performed at room temperature, and cells were kept in the dark when loaded. For cytoplasmic Ca\(^{2+}\) measurements, cells were loaded with Fura-2/AM (4 μM) for 40 min in an external solution comprised of the following: 145 mM NaCl, 2.8 mM KCl, 2 mM CaCl\(_2\), 2 mM MgCl\(_2\), 10 mM d-glucose, and 10 mM HEPES (pH 7.4 with NaOH). Calcium-free solution was of a similar composition as the external solution, except that 0.1 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid was substituted for calcium chloride. The number of calcium oscillations was quantified using IGOR Pro. Ca\(^{2+}\) spikes were considered to be oscillations when the R\(_{peak}\) – R\(_{base}\) value was >0.1.

Li\(^+\) Treatment—LiCl dissolved in DMEM replaced normal DMEM on cultured cells growing on coverslips. Cells were then maintained in the incubator for 40 min. Thereafter, cells were loaded with Fura-2/AM in the continuous presence of LiCl. Cells on the first coverslip were exposed to LiCl for 85–90 min prior to stimulation with LTC\(_4\). The last coverslip in the set had 110 min before stimulation.

Knockdown and cDNA Transfection Experiments—For siRNA and cDNA transfection, the Amaxa electroporation system was used (23) with the Amaxa cell line nucleofactor kit T.
Transfection efficiency, judged by GFP fluorescence following transfection with a GFP plasmid, was typically ~50–60%.

STIM1 siRNA was purchased from LifeTechnologies (catalog no. 4390815). Orai1 siRNA was bought from OriGene Technologies (catalog no. SR508429), and the sequences (5’ to 3’) were AGUGUACUCUGCUAAGGACGACGCG, CCAUAGACGGACCCAGAGUUCAGC, and AGGGAGAGUGUGGAAAGGAGG. Both STIM1 and Orai1 siRNAs were used at a final concentration of 50 nM.

PIPK1α and PIP5K1 γ siRNAs were from Dharmacon and used at a final concentration of 75 nM. The PIP5K1α sequences (5’ to 3’) were CGGCAAGAAACAUAGCAGCUUAG, GCCAGGCCCAAGCAUUA, GCCAUGAACGCCAAGAAACA, and GAAUAGGCCAUCAGG. The PIP5K1γ sequences (5’ to 3’) were UCUUGAGACGAGCAGUAA, GCCUCAUGCCAGGACGCUU, GAGAGAUGUGACAGCA, and GGAGAAGCCUGCAUCG.

Talin1 and talin2 siRNAs were from Dharmacon and used at 50 nM. The Talin1 SMARTpool sequences were CGGACCCAUUGCAGGUAUU, CGAUCGCCCGCAGAUUAU, GCCAUGAACCCGAAAACA, and GAAUAACCGCAUCAGG. The Talin2 SMARTpool sequences were UGUAGUACUCAGGCAAG, CCGGAAUGUAGGUGCAU, CAGGGACAAGCUUUGGCGGCA, and GCUGAAGACGGCAGG.

Immunofluorescence—Cells were fixed using 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100. Cells were then blocked using SuperBlock blocking buffer for 1 h at room temperature. Primary antibody (1:200) in PBS was added to the cells and left overnight at 4 °C. Cells were then washed at 10-min intervals in PBS with 0.1% Tween 20 (PBS-T) buffer. Alexa Fluor 488 anti-mouse or anti-rabbit or Alexa Fluor 568 anti-rabbit conjugated antibody was then added for 1 h at room temperature. Cells were mounted onto microscope slides using Vectashield antifade mounting medium with DAPI. Images were obtained using the inverted Olympus FV1000 confocal system equipped with a motorized stage, using a ×60 oil objective of 1.3 numerical aperture and excitation at 488 or 568 nm. All images were grouped according to an image size of 640 × 480 and a step size of 4 μm along the z axis. Fluorescence intensities were analyzed for each treatment and normalized to the maximum measured fluorescence using ImageJ.

Reverse Transcriptase Polymerase Chain Reaction—QIAshredder was used for homogenization of cell lysate, and total RNA was extracted using the Qiagen RNeasy mini kit. The process of reverse transcription of 1 μg of RNA was achieved using the iScript cDNA synthesis kit. The produced cDNA was amplified utilizing GoTaq Green master mix. The product of the polymerase chain reaction was separated by electrophoresis on 2% agarose gel. Primers were synthesized by Sigma: Talin-1, 5’-TCGGAAGTTGGTTGGTGTAG-T’3’ (sense) and 5’-GAGAACCCGCAAATACAGC-3’ (antisense); Talin-2, 5’-GTGCGACCTAGAAGACGC-3’ (sense) and 5’-GGCTTTCTTGGATGACGTGG-3’ (antisense).

Western Blot Analysis—Cells were lysed in radioimmunoprecipitation assay lysis buffer supplemented with protease inhibitor mixture, 0.1% Triton X-100, 10 mm sodium metavanadate, and 1 mm PMSF. 15 μg of protein was loaded into 10% SDS-PAGE gel. The protein was next transferred into a nitrocellulose membrane using a semidyird protein transfer apparatus (Bio-Rad). 5% nonfat dry milk in phosphate-buffered saline was used to block the membrane. The blocked membrane was then incubated with either PIP5 kinase α or γ antibody (1:2000) for 2 h at room temperature. After washing, a secondary antibody (1:4000) was applied in 5% nonfat dry milk in phosphate-buffered saline solution for 2 h at room temperature. Visualization was accomplished by the use of enhanced chemiluminescence plus the Western blotting detection system. The relative band intensities were analyzed using ImageJ. All bands were normalized to the corresponding β-actin levels as a loading control.

Statistical Analysis—All experiments were performed on three independent occasions unless specified otherwise in the text. Independent sample groups were first assessed for normality and equality of variances. To compare single-group treatment, an unpaired Student’s t test or Mann-Whitney U test was used (StatsDirect v2.6.2, Sale, UK). Differences were considered significant at p < 0.05. All data are presented in the text and figures as mean ± S.E. (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001).

Results

Oscillations in [Ca2+], Are Sustained by Ca2+ Entry through CRAC Channels in RBL-2H3 Cells—Activation of cysteinyl leukotriene type I receptors with LTC4 in RBL-1 cells evokes a series of oscillations in [Ca2+], that are supported by Ca2+ entry through CRAC channels (9). We repeated these experiments in the RBL-2H3 cell line, which has a stronger secretory phenotype. Stimulation with LTC4 resulted in numerous oscillations in [Ca2+], that were sustained for the duration of the experiment (800 s, Fig. 1A), although the amplitude decreased gradually with time because of receptor desensitization (24). To see whether Ca2+ entry through CRAC channels was required to maintain the oscillatory response, we used both pharmacological and siRNA-based approaches. La3+ is an effective CRAC channel blocker (25) and inhibited the rate of thapsigargin-induced Ca2+ influx with an IC50 of ~300 nM (Fig. 1, B and C). Pre-exposure to 10 μM La3+ accelerated the rundown of the oscillations in [Ca2+], induced by LTC4 in 2 mM external Ca2+ -containing solution (Fig. 1D). The rate of rundown in La3+ was similar to that seen in cells challenged with LTC4 in the absence of external Ca2+ (Fig. 1E; aggregate data from several experiments are summarized in Fig. 1F). Similar results were obtained when 20 μM BTP2 was used to block CRAC channels instead (Fig. 1F).

To extend the pharmacological results, we knocked down either STIM1 or Orai1 using siRNA-based approaches. Although both proteins were robustly expressed in wild-type RBL-2H3 cells, knockdown significantly reduced expression (STIM1, Fig. 1, G and H; Orai1, Fig. 1, I and J). Oscillations in [Ca2+], evoked by LTC4 in the presence of external Ca2+ ran down rapidly after knockdown of either STIM1 or Orai1 (Fig. 1K), and the responses resembled those observed when agonist was applied in the absence of external Ca2+ (Fig. 1L).
Oscillations in \([\text{Ca}^{2+}]_i\) in Response to Leukotriene Receptor Stimulation Run Down in the Presence of Li\(^+\) — Li\(^+\) is an effective inhibitor of inositol monophosphatases and, therefore, should lead to gradual depletion of PIP\(_2\) levels (18). Preincubation with 10 mM LiCl accelerated the rundown of oscillations in \([\text{Ca}^{2+}]_i\) evoked by LTC\(_4\) in the presence of external Ca\(^{2+}\) (Fig. 2A). The effect of LiCl was concentration-dependent (Fig. 2B), and the dose-inhibition curve yielded an IC\(_{50}\) of 15 mM (Fig. 2C), close to the value reported for IMPase2 (19).

The extent of Ca\(^{2+}\) release from the stores evoked by stimulation with either thapsigargin (Fig. 2D) or ionomycin (Fig. 2E and F) in Ca\(^{2+}\)-free external solution was unaffected by pretreatment with even high concentrations (50 mM) of Li\(^+\). Store-operated Ca\(^{2+}\) entry, induced by readmission of external Ca\(^{2+}\) to cells treated with thapsigargin, was also unaffected by Li\(^+\) (Fig. 2D). Pretreatment with Li\(^+\) also had no inhibitory effect on the transient oscillatory cytoplasmic Ca\(^{2+}\) signals evoked by LTC\(_4\) when agonist was applied in Ca\(^{2+}\)-free solution (Fig. 2G).
aggregate data are summarized in Fig. 2H). Collectively, these results rule out an action of Li+ on store Ca2+ content, store-operated Ca2+ entry, and agonist-induced InsP3 production.

The time course of rundown of cytoplasmic Ca2+ oscillations by LiCl in our experiments is broadly similar to the decline in InsP3 levels reported in Chinese hamster ovary cells following muscarinic receptor stimulation (26, 27). In these latter studies, pretreatment with 5–10 mM LiCl for 30 min had no effect on the muscarinic receptor stimulation (26, 27). In these latter studies, stimulated either in the presence or absence of external Ca2+

In the blowfly salivary gland, a seminal study by Fain and Berridge (28) has demonstrated that application of inositol, could reflect a role for Ca2+ entry in the synthesis of PIP2

stored in the continuous presence of agonist. We therefore examined whether the presence of exogenous inositol could rescue the cytoplasmic Ca2+ oscillations in Li+-treated cells. Although oscillations in [Ca2+]i, evoked by LTC4 ran down in the presence of LiCl (Fig. 3, A and B), pre-exposure to 10 mM inositol for a few minutes prior to stimulation rescued the oscillations in [Ca2+]i, (Fig. 3A; aggregate data are shown in Fig. 3B and compared with the control response). Interestingly, addition of inositol to control cells prior to stimulation with LTC4 in the presence of external Ca2+ resulted in an increase in the frequency of oscillations in [Ca2+]i, after agonist was added (Fig. 3, B and C).

![Graphs of cytoplasmic Ca2+ oscillations](image)

Although inositol rescued oscillations in [Ca2+]i, in Li+-treated cells, Ca2+ entry through CRAC channels was still required to sustain the oscillatory response. Exposure to the CRAC channel blocker La3+ accelerated the rundown of cytoplasmic Ca2+ oscillations in Li+-treated cells despite the presence of inositol (Fig. 3D; aggregate data are shown in Fig. 3F). Similar results were obtained when BTP2 was used to block the CRAC channels instead (Fig. 3, E and F).

The rundown of oscillations in [Ca2+]i, in Li+-treated cells when CRAC channels are blocked, despite the presence of inositol, could reflect a role for Ca2+ entry in the synthesis of PIP2.
from inositol. Alternatively, loss of the oscillatory response might simply arise from compromised store refilling. To distinguish between these possibilities, we took advantage of a protocol that enables store refilling to occur but in the absence of Ca$^{2+}$ entry through CRAC channels. Cytoplasmic Ca$^{2+}$ oscillations can be sustained in the absence of external Ca$^{2+}$ when Ca$^{2+}$ extrusion across the plasma membrane is inhibited, for example with high (millimolar) concentrations of La$^{3+}$ (9, 10). Under these conditions, Ca$^{2+}$ released from the stores cannot be exported out of the cell and so is taken back into the ER, thereby maintaining store Ca$^{2+}$ content in readiness for the next oscillatory cycle in [Ca$^{2+}$]. Stimulation with LTC$_4$ in Ca$^{2+}$-free solution supplemented with 1 mM La$^{3+}$ resulted in the generation of numerous oscillations in [Ca$^{2+}$] (Fig. 3G). Preincubation with LiCl led to rundown of the cytoplasmic Ca$^{2+}$ oscillations (Fig. 3H). However, inositol no longer rescued the oscillatory response in Li$^{+}$-treated cells (Fig. 3H; aggregate data are shown in Fig. 3I). These results, therefore, reveal a major role for Ca$^{2+}$ influx through CRAC channels in replenishment of the PIP$_2$ pool from inositol.

**Inhibition of Phosphatidylinositol 4-Kinase Accelerates the Rundown of Agonist-driven Oscillations in [Ca$^{2+}$].** The preceding results suggest that Ca$^{2+}$ influx through CRAC channels helps replenish PIP$_2$ levels during cytoplasmic Ca$^{2+}$ oscillations. This would mean that significant amounts of PIP$_2$ are hydrolyzed following stimulation with LTC$_4$ and that, therefore, phosphatidylinositol 4- and 5-kinases should be active during stimulation. A simple prediction is therefore that inhibition of these kinases should accelerate the rundown of the oscillations in [Ca$^{2+}$], because of the loss of PIP$_2$ production. At relatively high concentrations, wortmannin inhibits phosphatidylinositol 4-kinase (29, 30) and, therefore, impairs PIP$_2$ resynthesis. Compared with control responses, pretreatment with 10 $\mu$M wortmannin resulted in faster rundown of cytoplasmic Ca$^{2+}$ oscillations evoked by LTC$_4$ (compare the control response in Fig. 4A with that evoked in the presence of wortmannin in Fig. 4B). The number of Ca$^{2+}$ oscillations that were triggered by LTC$_4$ in the presence of wortmannin (Fig. 4C) was similar to the number seen in Ca$^{2+}$-free solution (Fig. 1L). A previous study reported that wortmannin inhibited store-operated Ca$^{2+}$ entry through inhibition of phosphatidylinositol 4-kinase and, thereby, depletion of PIP$_2$ (31). In that report, a prominent inhibition of Ca$^{2+}$ influx was seen when wortmannin pretreatment was combined with exposure to the agonist methacholine to ensure PIP$_2$ depletion. Pretreatment with wortmannin (20 $\mu$M) but in the absence of methacholine had no inhibitory effect on Ca$^{2+}$ entry (31). These data are consistent with our previous report showing that wortmannin alone had no effect on CRAC channel activity in RBL cells (32). Furthermore, loss of cytoplasmic Ca$^{2+}$ oscillations evoked by LTC$_4$ in the presence of wortmannin could be rescued by exogenous PI4P (Fig. 4, B and C), consistent with an action by wortmannin on phosphatidylinositol 4-kinase. Wortmannin also blocks phosphatidylinositol 3-kinase. However, pretreatment with...
the PI 3-kinase inhibitor LY294002 did not mimic the inhibitory effect of wortmannin on oscillations evoked by LTC₄ (Fig. 4C).

**Application of Phosphatidylinositol 4-Phosphate Rescues Oscillations in [Ca²⁺]ᵢ Evoked by LTC₄ in Li⁺-treated Cells—**

The results described above with wortmannin suggest that PI4P production is important for sustaining oscillations in [Ca²⁺]ᵢ, evoked by LTC₄. To test this more directly, we applied purified PI4P to Li⁺-treated cells to see whether this could prevent rundown of the cytoplasmic Ca²⁺ oscillations. In control cells not treated with LiCl, exposure to PI4P led to a small increase in the number of oscillations in [Ca²⁺]ᵢ over an 800-s recording period for the different conditions are shown. La³⁺ in 0 mM Ca²⁺ denotes 0 Ca²⁺ supplemented with 1 mM La³⁺. **p < 0.0001.

**Knockdown of PI5 Kinase I Abolishes Oscillations in [Ca²⁺]ᵢ Evoked by LTC₄**—

PI4P is converted to PI₅P by type I PI5 kinases, which consist of three isoforms: PI5 kinase 1α, 1β, and 1γ. RT-PCR revealed the presence of PI5 kinase 1α and 1γ in RBL-2H3 cells but not the 1β isoform (Fig. 5, A and B). The presence of these proteins was confirmed by immunocytochemistry (Fig. 5, C and D) and Western blots (Fig. 5, E and F). SiRNA against either PI5 kinase 1α or PI5 kinase 1γ resulted in an ~60% reduction in protein expression (Fig. 5, C–F). Knockdown of PI5 kinase 1α had no effect on the expression of PI5 kinase 1γ and vice versa (Fig. 5G). Following knockdown of PI5 kinase 1α, the typical oscillatory response in [Ca²⁺]ᵢ evoked by LTC₄ in the presence of external Ca²⁺ was abolished (Fig. 5, H and J). Similar results were observed when PI5 kinase 1γ was knocked down instead (Fig. 5, I and J), suggesting redundancy between these isoforms. Knockdown of either PI5 kinase isoform had only a modest effect on responses in Ca²⁺-free solution (Fig. 5, K–M). Neither the store Ca²⁺ content, as measured by the response to thapsigargin in Ca²⁺-free solution, nor the rate of store-operated Ca²⁺ influx were affected by knockdown of either PI5 kinase isoform (Fig. 5, N and O).
Oscillations in [Ca$^{2+}$]i in Li$^{+}$-treated cells challenged with LTC$_4$ in the presence of external Ca$^{2+}$ run down within a few minutes (Fig. 2, A and B) but can be rescued by PI4P (Fig. 4, E and I). To see whether this rescue required conversion of PI4P to PIP2, we knocked down either PIP5 kinase 1α or 1β and examined whether this prevented PI4P from rescuing the cytoplasmic Ca$^{2+}$ oscillations evoked by LTC$_4$. In Li$^{+}$-treated cells in which PIP5 kinase 1α or 1β had been knocked down, oscillations in [Ca$^{2+}$]i ran down within 5 min (Fig. 6, A–C). However, under these conditions, application of PI4P no longer rescued the cytoplasmic Ca$^{2+}$ oscillations (Fig. 6, D–F).

**FIGURE 5.** PIP5K1 isoforms are involved in maintaining LTC$_4$-driven cytoplasmic Ca$^{2+}$ oscillations. A, RT-PCR comparing the expression of PIP5K1 isoforms in RBL-2H3 cells. B, PIP5K1β mRNA is absent from RBL-2H3 cells despite genomic DNA (gDNA) being detectable. C and D, confocal microscopy images comparing the expression of PIP5K1α and β protein between control cells and those in which either PIP5K1α (C) or PIP5K1β (D) had been knocked down (KD). The corresponding histograms summarize data from >40 cells in each group. ****, p < 0.0001. E, Western blot comparing the expression of PIP5K1α in control (Ctrl) cells and after siRNA-directed knockdown. *, p < 0.05. F, Western blot comparing the expression of PIP5K1 β in control cells and after siRNA-directed knockdown. *, p < 0.05. G, Western blot comparing the expression of PIP5K1α after knockdown of PIP5K1γ (top panel) and vice versa (bottom panel). H and I, cytoplasmic Ca$^{2+}$ oscillations evoked by LTC$_4$, run down quickly after knockdown of PIP5K1α (H) or PIP5K1β (I). Scrambled siRNA controls are included. J, histogram comparing the total number of cytoplasmic Ca$^{2+}$ oscillations evoked by LTC$_4$ in 2 mM external Ca$^{2+}$ over 800 s of stimulation from three independent experiments. ****, p < 0.0001. K and L, the effect of knockdown of PIP5K1α (K) or PIP5K1β (L) on responses evoked by LTC$_4$ in Ca$^{2+}$-free solution. M, histogram comparing the average number of Ca$^{2+}$ oscillations in Ca$^{2+}$-free solution for the conditions shown. ***, p < 0.001. N, Ca$^{2+}$ release and Ca$^{2+}$ influx evoked by thapsigargin are compared following knockdown of PIP5K1α and PIP5K1β or after transfection with scrambled siRNA. O, the rates of Ca$^{2+}$ entry following stimulation with thapsigargin as in K are compared for the conditions shown. ns, not significant.
Prolonged Agonist-evoked Oscillations in [Ca\(^{2+}\)] \(\text{cyt}\) Require Talin1—To identify a potential mechanism linking Ca\(^{2+}\) influx to PIP\(_2\) replenishment, we sought Ca\(^{2+}\)-dependent proteins that regulate PIP5 kinase activity. One candidate is talin, an adaptor protein that links integrin to the actin cytoskeleton (35). There are two homologous talin isoforms, talin1 and talin2. The NH\(_2\)-terminal globular head has a band4.1/ezrin/radixin/moesin-like domain that binds actin, PIP\(_2\), and PIP5 kinase (35, 36). Talins can be cleaved by the Ca\(^{2+}\)-dependent protease calpain (37), providing a possible link between cytoplasmic Ca\(^{2+}\) and PIP5 kinase activity. Talin1 mRNA was expressed in RBL-2H3 cells, whereas talin2 was undetectable (Fig. 7A). Immunocytochemical studies confirmed the presence of talin1 and, this was reduced by \(\sim 50\%\) following knockdown (Fig. 7B). Oscillations in [Ca\(^{2+}\)] \(\text{cyt}\) evoked by LTC\(_4\) ran down considerably more quickly following talin1 knockdown (Fig. 7C-D). Neither store-operated Ca\(^{2+}\) influx, measured by stimulating cells with thapsigargin (Fig. 7E), nor Ca\(^{2+}\) release in the absence of Ca\(^{2+}\) influx (Fig. 7F) were impaired following talin1 knockdown. Pre-exposure to either inositol (Fig. 7G) or PIP4P (Fig. 7H) did not rescue cytoplasmic Ca\(^{2+}\) oscillations to LTC\(_4\) following knockdown of talin1. Aggregate data from several such experiments are summarized in Fig. 7J.

Independence of P2Y and cysLT1 Receptors—CysLT1 receptor-dependent Ca\(^{2+}\) signaling is affected by the presence of caveolin-1 and is disrupted by removal of cholesterol from the plasma membrane by methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD), suggesting the leukotriene receptors signal within lipid raft domains (23).

In RBL-1 cells, stimulation of P2Y receptors with ATP generates InsP\(_3\), which then triggers Ca\(^{2+}\) release from the stores. P2Y-dependent responses are unaffected by M\(\beta\)CD, indicating that they are not located close to cysLT1 receptors (23). To test whether P2Y and cysLT1 receptors coupled to the same pool of PIP\(_2\), we designed experiments to see whether ATP was able to evoke InsP\(_3\)-dependent Ca\(^{2+}\) release after responses to LTC\(_4\) had ceased. We first confirmed that previous findings made in RBL-1 cells also occurred in the RBL-2H3 cell line. Immunocytochemical studies showed expression of cysLT1 receptors in the cell periphery, and this was not altered by M\(\beta\)CD (Fig. 8A). Oscillations in [Ca\(^{2+}\)] \(\text{cyt}\) evoked by LTC\(_4\) were abolished by M\(\beta\)CD (Fig. 8, B and C). However, ATP still elicited a large Ca\(^{2+}\) transient in the presence of M\(\beta\)CD (Fig. 8D).

After cytoplasmic Ca\(^{2+}\) oscillations evoked by LTC\(_4\) had run down in Li\(^{+}\)-treated cells, we applied ATP to see whether Ca\(^{2+}\) release could still be evoked. A robust Ca\(^{2+}\) transient occurred (Fig. 8, E and G), revealing that P2Y receptors were able to couple to phospholipase C under conditions where cysLT1 receptors could not.

To ensure that there was no PIP\(_2\) resynthesis during the interval between LTC\(_4\) exposure and ATP stimulation, we pretreated cells with both LiCl and wortmannin. After oscillations in [Ca\(^{2+}\)] \(\text{cyt}\), following challenge with LTC\(_4\) had run down (Fig. 8F), subsequent exposure to ATP elicited robust Ca\(^{2+}\) release (Fig. 8, F and G).

Discussion

Our findings reveal a role for Ca\(^{2+}\) entry through CRAC channels in regulating PIP\(_2\) replenishment and, therefore, in supporting oscillations in [Ca\(^{2+}\)] \(\text{cyt}\), following physiological levels of cysLT1 receptor stimulation. A schematic summarizing our main findings and the sites of intervention is shown in Fig. 9.

Cytoplasmic Ca\(^{2+}\) oscillations evoked by LTC\(_4\) ran down in the presence of Li\(^{+}\), an inhibitor of inositol monophosphatases (18), demonstrating that PIP\(_2\) resynthesis was required for maintaining the Ca\(^{2+}\) response. Direct application of either inositol or phosphatidylinositol 4-phosphate was able to rescue the oscillatory Ca\(^{2+}\) response in the presence of Li\(^{+}\), consistent with an action of Li\(^{+}\) on inositol monophosphatase. However, recovery of the oscillations in [Ca\(^{2+}\)] \(\text{cyt}\) evoked by LTC\(_4\) was prevented by block of CRAC channel activity. Our results suggest that Ca\(^{2+}\) influx is required to replenish the PIP\(_2\) pool that is targeted by cysLT1 receptors. Phosphatidylinositol 4-phosphate is phosphorylated to PIP\(_2\) by three isoforms of the type I PIP5 kinase (33) that are localized to various membrane compartments, including the plasma membrane, through a dileucine motif and another conserved lysine within the activation loop, a...
**Store-operated Ca\(^{2+}\) Entry Regulates PIP\(_{2}\) Levels**

Figure 7. Knockdown of talin1 accelerates the rundown of oscillations in \([Ca^{2+}]\) evoked by LTC\(_4\).

A, RT-PCR reveals the presence of talin1 but not talin2 in RBL-2H3 cells. B, immunocytochemical detection of talin1 in control cells and after knockdown (KD). The histogram summarizes data from between 40 and 52 cells for each condition. ***, p < 0.0001. C, cytoplasmic \([Ca^{2+}]\) oscillations evoked by LTC\(_4\) rundown more quickly after talin1 knockdown. D, aggregate data from several experiments are summarized. Each column represents data from between 24 and 32 cells. E, thapsigargin-evoked cytoplasmic \([Ca^{2+}]\) signals are unaffected by talin1 knockdown. F, the total number of cytoplasmic \([Ca^{2+}]\) oscillations evoked by LTC\(_4\) in Ca\(^{2+}\)-free solution are similar in control cells and those in which talin1 had been knocked down. ns, not significant. G, oscillations in \([Ca^{2+}]\) evoked by LTC\(_4\) in talin1-deficient cells are not rescued by addition of inositol. Control refers to a wild-type cell stimulated with LTC\(_4\) in the presence of inositol. H, PI4P fails to rescue cytoplasmic \([Ca^{2+}]\) oscillations in cells following knockdown of talin1. I, aggregate data from several independent experiments are compared. ****, p < 0.0001 compared with the corresponding controls. LTC\(_4\) was used to stimulate the cells. KD, knockdown of talin1.

stretch of ~20 amino acids in the C terminus (38). RBL-2H3 cells expressed both PIP5 kinase \(\alpha\) and \(\gamma\), and knockdown of either accelerated rundown of the cytoplasmic \(Ca^{2+}\) oscillations evoked by LTC\(_4\). The simplest explanation for our results is, therefore, that \(Ca^{2+}\) entry through CRAC channels stimulates PIP5 kinase \(\alpha/\gamma\) to convert phosphatidylinositol 4-phosphate to PIP\(_2\) and, therefore, ensure adequate levels of the phospholipid for sustained cysLT1 receptor-dependent \(Ca^{2+}\) influx. For example, \(Ca^{2+}\) influx might also stimulate PI transfer from the peripheral ER to the plasma membrane, where phosphatidylinositol transfer proteins, including Nir2, shuttle PI from the ER to the plasma membrane (39). Although such a mechanism might contribute to PIP\(_2\) replenishment under our conditions, our data suggest that the key step that is regulated by \(Ca^{2+}\) influx following cysLT1 receptor activation is the conversion of phosphatidylinositol 4-phosphate to PIP\(_2\) by PIP5 kinase, a reaction that takes place mainly in the plasma membrane.

The frequency of \(InsP_{3}\)-dependent cytoplasmic \(Ca^{2+}\) oscillations can be altered by varying the rate or extent of \(Ca^{2+}\) entry (40). We now find that oscillation frequency evoked by LTC\(_4\) can also be increased by supply of exogenous inositol or PI4P. This suggests that PIP\(_2\) availability is rate-limiting. Providing more precursor accelerates its replenishment, and this increases the frequency of oscillations in \([Ca^{2+}]\).

Growing evidence points to the existence of local pools of PIP\(_2\) within the plasma membrane (41). Our functional studies are consistent with this view and suggest that these local pools exchange slowly. Oscillations in \([Ca^{2+}]\) evoked by cysLT1 receptor activation ran down quickly in the presence of Li\(^{+}\) and wortmannin, which inhibit inositol monophosphatases and PI4P kinases, respectively. Nevertheless, subsequent stimulation of P2Y receptors, which also couple to phospholipase C\(\beta\), elicited \(Ca^{2+}\) release that was similar in size and kinetics to responses obtained in the absence of the inhibitors. Therefore, P2Y receptors access a PIP\(_2\) pool that is distinct from that utilized by cysLT1 receptors.
In summary, our findings identify a role for Ca\(^{2+}\) entry through CRAC channels in maintaining a plasma membrane pool of PIP\(_2\) that is accessible to cysLT1 receptors. Ca\(^{2+}\) influx, therefore, has two important roles in controlling cytoplasmic Ca\(^{2+}\) oscillations: maintaining PIP\(_2\) levels and refilling the intracellular Ca\(^{2+}\) stores. The former will be important in initiating each oscillation in \([\text{Ca}^{2+}]_i\), whereas the latter will help set the interspike interval. Both will affect spike frequency but through distinct mechanisms. Stimulation of PIP\(_2\) production constitutes an interesting autoregulatory mechanism through which CRAC channels will be able to sustain their activity by ensuring that there are sufficient PIP\(_2\) levels in the plasma membrane to produce InsP\(_3\) and, therefore, store depletion, which is required for the opening of the channels. How Ca\(^{2+}\) influx activates PIP5 kinase and whether this is driven by local Ca\(^{2+}\) entry requires further investigation.

**Author Contributions**—A. A. and A. B. P. designed the study. A. A. performed and analyzed the experiments. A. B. P. wrote the manuscript. Both authors approved the final version of the manuscript.

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Ca^{2+} Influx through Store-operated Calcium Channels Replenishes the Functional Phosphatidylinositol 4,5-Bisphosphate Pool Used by Cysteinyl Leukotriene Type I Receptors

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