Rapid Communication

Agonist-Induced Calcium Entry Correlates With STIM1 Translocation

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The mechanisms of agonist-induced calcium entry (ACE) following depletion of intracellular calcium stores have not been fully established. We report here that calcium-independent phospholipase A (iPLA2) is required for robust Ca2+-entry in HaCaT keratinocytes following ATP or UTP stimulation. Lysophosphatidic acid (LPA), an unrelated agonist, evoked Ca2+ release without inducing robust Ca2+-entry. Both LPA and UTP induced the redistribution of STIM1 into puncta which localized to regions near or at the plasma membrane, as well as within the cytoplasm. Plasma membrane-associated STIM1 remained high for up to 10 min after UTP stimulation, whereas it had returned almost to baseline by that time point in LPA-stimulated cells. This correlated with faster reloading of the endoplasmic reticulum Ca2+-stores in LPA-treated cells. Thus by differentially regulating store-refilling after agonist-mediated depletion, LPA and UTP may exert distinct effects on the duration of STIM1 localization at the plasma membrane, and thus, on the magnitude and duration of ACE.

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Abbreviations: ACE, agonist-induced Ca2+-entry; BEL, bromoeno lactone; [Ca2+]i, extracellular Ca2+; [Ca2+]j, free cytosolic Ca2+; CIF, Ca2+ influx factor; DAG, diacglycerol; ER, endoplasmic reticulum; ICa2+, Ca2+ release-activated Ca2+ current; iPLA2, Ca2+-independent phospholipase A2; KHB, Krebs-Henseleit buffer; PM, plasma membrane; SOCE, store-operated Ca2+ entry; TG, thapsigargin.

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Tight control of free cytosolic calcium ([Ca2+]i) enables this second messenger to regulate diverse cell processes (Berridge et al., 2000). Receptor-mediated activation of phospholipase C (PLC) stimulates hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (Berridge et al., 2003). The latter evokes Ca2+ release via IP3 receptors (IP3Rs) on the endoplasmic reticulum (ER). This is usually followed by Ca2+ influx across the plasma membrane (PM), or agonist-induced Ca2+ entry (ACE) (Patterson et al., 2002). Similarly, depletion of ER stores by inhibition of the sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) with thapsigargin (TG) evokes store-operative Ca2+ entry (SOCE) (Parekh and Putney, 2005).

A mechanism for SOCE proposed by Bolotina and colleagues (Smani et al., 2004) involves a 600-Da diffusible factor “calcium influx factor” (CIF) of unknown identity that is released from the ER following store depletion (Randriamampita and Tsien, 1993; Bolotina and Csutora, 2005). CIF in turn activates calcium-independent phospholipase A2 (iPLA2) which generates lysophospholipids that activate SOCE at the PM by an uncharacterized process. In their studies, SOCE was impaired by knockdown of iPLA2 using RNA interference or by treatment with an iPLA2 inhibitor (Smani et al., 2004). Notably, STIM1 also appears to associate with and activate TRPC1, a member of transient receptor potential family of cation entry channels (Huang et al., 2006; Lopez et al., 2006).

In this study, we have examined the role of iPLA2 and STIM1 in ACE in HaCaT keratinocytes. We have found that inhibition of iPLA2 with BEL impaired UTP and ATP-induced Ca2+ entry. We have also observed that stimulation with physiological agonists triggered restructuring of STIM1 into puncta that were assembled at or near the PM. The duration of STIM1 localization to the PM appeared to be agonist dependent, with UTP promoting sustained targeting of STIM1 to the PM whereas lysophosphatidic acid (LPA) induced only transient association of STIM1 with the PM. Together, our findings suggest that
differential signaling pathways differentially regulate ACE by
controlling the duration of STIM1 localization to the PM.

Materials and Methods

Reagents

Fluo-4-AM was obtained from Invitrogen (Paisley, UK), bromoenoel lactone (BET) from Sigma (Poole, Dorset, UK). All other reagents, including MCDB153 medium were from Sigma unless indicated otherwise. The dsRed-ER vector was from Clontech (Mountain View, CA).

Cell culture and nucleofection

HaCaT keratinocytes, a kind gift from Dr. NE Fusenig (German Cancer Research Center, Heidelberg), were grown in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics. The YFP-STIM1 expression construct was a generous gift from Tobias Meyer (Stanford University, Stanford, CA). Cells were nucleofected (Amaxa Biosystems, Cologne, Germany) according to the manufacturer’s instructions. Briefly, 10^6 cells were resuspended in 100 µl of nucleofection solution with 5 µg of YFP-STIM1 plasmids, transferred to a cuvette and nucleofected at room temperature. The cells were re-suspended in 500 µl of complete medium and seeded in Wilco glass-bottomed microwell dishes (Intracel, Royston, UK). In some experiments, transfections were performed with Lipofectamine Plus (Invitrogen (Paisley, UK)) according to the manufacturer’s protocol.

Calcium imaging

HaCaT keratinocytes were seeded in Wilco glass-bottomed microwell dishes (Intracel, Royston, UK) the day prior to experimentation. Cells were loaded with 3 µM of Fluo-4 acetoxymethyl (AM) ester for 45 min in supplemented MCDB153 medium (Todd and Reynolds, 1998) with 70 µM Ca^{2+} unless indicated otherwise. To minimize compartmentalization of the dye, 200 µM of the anion transport inhibitor sulphinpyrazone in dimethylsulphoxide (DMSO) was included in the medium during loading and de-esterification (Di Virgilio et al., 1988). After loading, cells were washed in phosphate-buffered saline (PBS) and incubated in medium for 45–60 min at 37°C to allow de-esterification of the dye. Vehicle, or BET at 10–20 µM, was added to the medium at a final concentration of 10–20 µM for the last 30 min of de-esterification. Fluorescence quenching assays were performed by adding MnCl_{2} (prepared in PBS) at a final concentration of 500 µM. For Ca^{2+} -free assays, cells were loaded as above using nominally Ca^{2+}-free 200 mmol/L Heps-HCl buffer. Changes in [Ca^{2+}], were monitored at 4-sec intervals with a Leica TCS SP2 confocal laser scanning microscope equipped with an argon laser (Leica, Milton Keynes, UK). A heated stage was used to maintain the cells at 37°C during image acquisition, and images were captured using a 63× Plan Apo objective (NA1.32). Fluo-4 was excited with the 488-nm line of the laser, collecting emitted fluorescence through a 500–550 nm window of the detector. Quantification was performed with Leica confocal software, and changes in [Ca^{2+}] were expressed as the ratio of the initial fluorescence to the temporal fluorescence (F_0/F_0).

Analysis of STIM1 translocation

The cells were washed 2–3 times in nominally Ca^{2+}-free KHb prior to visualization. Images of YFP-STIM were then acquired at a final concentration of 500 µM. For Ca^{2+}-free assays, cells were loaded as above using nominally Ca^{2+}-free 200 mmol/L Heps-HCl buffer. Changes in [Ca^{2+}], were monitored at 4-sec intervals with a Leica TCS SP2 confocal laser scanning microscope equipped with an argon laser (Leica, Milton Keynes, UK). A heated stage was used to maintain the cells at 37°C during image acquisition, and images were captured using a 63× Plan Apo objective (NA1.32). Fluo-4 was excited with the 488-nm line of the laser, collecting emitted fluorescence through a 500–550 nm window of the detector. Quantification was performed with Leica confocal software, and changes in [Ca^{2+}] were expressed as the ratio of the initial fluorescence to the temporal fluorescence (F_0/F_0).

Statistical analysis

Results of the Ca^{2+} imaging experiments are presented as means (±SEM) which were determined in GraphPad Prism or Microsoft Excel. Statistical significance was determined using the unpaired two-tailed Student’s t-test (GraphPad Prism or Microsoft Excel). Results with P < 0.05 were considered significant.

Results

UTP induces Ca^{2+} entry in keratinocytes

Extracellular nucleotides enhance proliferation of keratinocytes and other cells (Burrell et al., 2003). Stimulation of HaCaT keratinocytes with ATP or UTP induce[s] [Ca^{2+}], oscillations of increasing amplitude in the absence of external Ca^{2+} (Burrell et al., 2003). With only 70 µM extracellular Ca^{2+} ([Ca^{2+}]), in the medium, we observed that stimulation with UTP evoked a rapid increase in [Ca^{2+}], that remained elevated for the duration of recording (Fig. 1A), suggesting that UTP induced Ca^{2+} entry. To confirm that the sustained [Ca^{2+}], elevation was due to Ca^{2+} entry (and not for instance inhibition of Ca^{2+} pumps), paired assays were performed in which cells were stimulated with UTP alone or UTP and the Ca^{2+} chelator EGTA. Simultaneous addition of UTP and EGTA resulted in a gradual return of the [Ca^{2+}], to basal levels (Fig. 1B), indicating that chelation of [Ca^{2+}], abolishes the sustained [Ca^{2+}], plateau. Similar results were obtained with ATP (data not shown).

To provide further evidence that the agonist-induced elevation of [Ca^{2+}], was due to Ca^{2+} entry across the PM, we performed fluorescence quenching assays in which Mn^{2+} was added to the medium after stimulation. Addition of Mn^{2+} led to a return of the [Ca^{2+}], signal baseline levels (Fig. 1C). Taken together, these data confirm that stimulation of HaCaT keratinocytes with UTP results in Ca^{2+} entry across the plasma membrane.

Inhibition of iPLA{sub}2, impairs ACE

Inhibition of iPLA{sub}2 with BEL, a specific pharmacological inhibitor with a 1000-fold selectivity for iPLA{sub}2 over cytosolic (85 kDa) PLA{sub}2 (Hazan et al., 1991) has been shown to impair SOCE (Smami et al., 2004). However, electrophysiological measurements suggest that TG-induced SOCE might not be mediated by the same channels that mediate IP_{3}-activated SOCE (Vanden Abeele et al., 2004). Therefore, we investigated whether iPLA{sub}2, activity was required for ACE. As shown in Figure 2A, treatment of cells with BEL led to a rapid decline in the [Ca^{2+}], signal following stimulation with UTP, whereas [Ca^{2+}], remained elevated in control cells. Similar results were obtained when the cells were stimulated with ATP (Fig. 2B). In addition to inhibiting iPLA{sub}2, BEL has also been reported to inhibit Mg^{2+}-dependent phosphatidate phosphohydrolase (PAP-1), an enzyme involved in DAG turnover (Balsinde and Dennis, 1996). Treatment of cells with the PAP-1 inhibitor, propranolol (150 µM, 30 min preincubation (Fuentes et al., 2003), did not impair ACE (data not shown). Thus the effects of BEL on ACE can be attributed to its inhibition of iPLA{sub}2 and not PAP-1.

LPA induces Ca^{2+} release but not sustained Ca^{2+} entry

LPA evokes Ca^{2+} release in many cells and modulates keratinocyte growth and migration (Mills and Moolenaar, 2003; Sauer et al., 2004). We stimulate cells with LPA to determine if it evoked Ca^{2+} entry. As shown in Figure 3, although Ca^{2+} release was observed, this was not followed by an elevated plateau, even though the extent of Ca^{2+} release (initial Ft/F0 peak) was similar to that obtained with UTP and ATP (compare Figs. 3A with 2A). Thus LPA does not appear to induce significant Ca^{2+} influx in HaCaT keratinocytes. We have obtained similar results on primary normal epidermal keratinocytes. The same observations have been made on T cells and fibroblasts (Takemura et al., 1996; Waldron et al., 1997). The inability of LPA to stimulate robust Ca^{2+} entry compared to UTP was not due to differences in agonist

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potency, since a dose response assay showed that UTP and LPA were essentially equipotent (Fig. 3B), with EC\textsubscript{50} values of 4.0 nM and 5.4 nM respectively.

**STIM1 translocation**

Recently, STIM1 has been identified as a key mediator of SOCE (Liou et al., 2005; Zhang et al., 2005). When YFP-STIM1 was expressed in HaCaT keratinocytes, a reticular distribution was observed (Fig. 4A), similar to the pattern observed in other cell types (Liou et al., 2005). Co-expression of an ER marker (dsRed-ER) confirmed that YFP-STIM1 localized predominantly to the ER (Fig. 4B). However, YFP-STIM1 was also observed in regions where little or no ER staining was detected, notably at the extremities of the cells. Thus in resting cells, YFP-STIM1 appears to localize both to the ER and to other subcellular domains.

Next we tested the ability of UTP and LPA to promote STIM1 redistribution. These experiments were performed in Ca\textsuperscript{2+}-free buffer to avoid any potential effects of Ca\textsuperscript{2+} entry itself on the spatiotemporal dynamics of STIM1. Both agonists induced the assembly of YFP-STIM1 puncta, which were formed within the cytoplasm and also at PM (Fig. 4C, D). This pattern is consistent with that observed by Meyer and colleagues on HeLa cells treated with histamine and thapsigargin (Liou et al., 2005). The lifetimes of the puncta were generally shorter upon LPA stimulation compared to UTP stimulation (compare Fig. 4C, D). Consistent with this, PM-proximal puncta levels continued to rise for almost 10 min after UTP stimulation (Fig. 4E). In contrast, YFP-STIM1 was not retained at the PM of LPA-treated cells. Instead, after reaching a peak about 3 min after stimulation, YFP-STIM1 appeared to return to the cytoplasm, such that by 10 min PM-associated YTP-STIM1 had returned to near-baseline levels. Thus the differential abilities of UTP and LPA to promote Ca\textsuperscript{2+} entry appear to be related to STIM1 translocation.

**Differential store refilling**

According to the current model, STIM1 is held predominantly in the ER with its unpaired Ca\textsuperscript{2+}-binding EF hand in the ER lumen. Store depletion causes dissociation of Ca\textsuperscript{2+} from the EF hand, evoking STIM1 translocation. Our findings suggested that STIM1 starts migrating from the PM back to the ER shortly after LPA treatment, and this correlates with a reduction in Ca\textsuperscript{2+} entry (see Figs. 4D and 3A). Given that store refilling is thought to contribute to deactivation of Ca\textsuperscript{2+} influx and I\textsubscript{CRAC} (Parekh and Putney, 2005), we speculated that Ca\textsuperscript{2+} stores may be reloaded more rapidly post-LPA stimulation compared to UTP stimulation. To test this hypothesis, cells were stimulated with agonists in a nominally Ca\textsuperscript{2+}-free buffer. After the initial transient had returned to baseline, the cells were then treated with TG to empty the stores of residual Ca\textsuperscript{2+}. As shown in Figure 5, the TG-induced Ca\textsuperscript{2+} peak after LPA treatment was significantly higher than that obtained after UTP. Thus by differentially regulating the re-filling of the Ca\textsuperscript{2+} stores,
Fig. 2. Pharmacological inhibition of iPLA₂ impairs ACE. Averaged traces from paired assays showing the mean changes in $[\text{Ca}^{2+}]_i$ in HaCaT keratinocytes treated with the iPLA₂ inhibitor BEL (filled circles), or with vehicle (open circles) for 30 min before imaging. The cells were stimulated with (A) 10 μM UTP, or (B) 10 μM ATP, as indicated. Numbers of cells (n) averaged: (A) BEL 35, DMSO 34; (B) BEL 25, DMSO 23. (C, D) Summary data pooled from three to four independent experiments (n = 104–135 cells). Plateau phases were averaged over 100 sec.

Fig. 3. LPA-induces $[\text{Ca}^{2+}]_i$ release but not robust $\text{Ca}^{2+}$ entry. A: HaCaT keratinocytes were stimulated with 10 μM LPA at the time point indicated by the arrow. Trace shown was averaged from n = 32 cells from one experiment, and similar results were obtained in four independent experiments. B: Dose-response curves for UTP (open circles) and LPA (filled squares). Results are the means ± SEM of the peak F/F₀ ratio determined from 26 to 46 cells.
physiological agonists appear to control the duration of STIM1 localization to the PM and thus the magnitude and duration of ACE.

Discussion

The activation of \( \text{Ca}^{2+} \) entry is the predominant mechanism for sustained elevation of \([\text{Ca}^{2+}]_{\text{i}}\), in non-excitable cells. The fundamental elements of SOCE are only now beginning to be defined. Studies by various groups have indicated that iPLA\(_2\) activity is required for SOCE (Smani et al., 2004; Vanden Abeele et al., 2004). In the present work, we have shown that iPLA\(_2\) activity is required for sustained ACE in HaCaT keratinocytes stimulated with extracellular nucleotides. Although experimental store depletion is often achieved by inhibition of the SERCA pump with TG, our observations indicate that iPLA\(_2\)-mediated \( \text{Ca}^{2+} \) influx is likely to be functional in response to physiological agonists, not just TG. Thus even though Pevarskaya and colleagues found that the \( \text{Ca}^{2+} \) current generated by store depletion with TG was significantly more sensitive to BEL treatment than that generated by IP\(_3\) (Vanden Abeele et al., 2004), we argue in the present study that robust ACE in HaCaT keratinocytes is dependent on iPLA\(_2\). Both arachidonic acid and lysophospholipid products of iPLA\(_2\) activity have been implicated in \( \text{Ca}^{2+} \) entry (Smani et al., 2004; Mignen et al., 2005). However, arachidonic acid-mediated \( \text{Ca}^{2+} \) influx seems to function predominantly at low agonist concentrations (Shuttleworth et al., 2004). Therefore, given that our experiments were performed with supramaximal agonist concentrations of 10 \( \mu \text{M} \) ATP or UTP (see Fig. 3B), it is unlikely that \( \text{Ca}^{2+} \)-selective arachidonate-regulated channels were significantly active.

Extracellular nucleotides signal through the P2Y family of G protein-coupled receptors (GPCR) (White and Burnstock, 2006). In addition, ATP also activates the P2X family of ion channels (White and Burnstock, 2006). However, given the similarity in the \([\text{Ca}^{2+}]_{\text{i}}\) dynamics of ATP and UTP-treated cells, the contribution of P2X channels to ATP-induced \( \text{Ca}^{2+} \) entry under our experimental conditions was arguably minimal.

In our investigations, we found that UTP and ATP evoked greater \( \text{Ca}^{2+} \) entry compared to LPA. This suggested that even though the respective UTP and LPA signaling pathways were
equipotent for Ca\(^{2+}\) release on HaCaT keratinocytes (Fig. 3B), they were differentially coupled to Ca\(^{2+}\) entry. Given the recent identification of STIM1 as a Ca\(^{2+}\) sensor in the ER, we speculated that this might be related to divergent effects on the spatiotemporal dynamics of STIM1. Examination of YFP-STIM1 kinetics in the absence of [Ca\(^{2+}\)]\(_{o}\) revealed striking puncta formation at the PM as well as in the cytoplasm. Exogenous UTP or LPA promoted translocation of YFP-STIM1 to the PM in the absence of [Ca\(^{2+}\)]\(_{o}\), confirming that STIM1 translocation is likely to be a cause rather than consequence of ACE. The bulk of YFP-STIM1 persisted at the PM for up to 10 min following UTP stimulation (Fig. 4C,E) and in some experiments, up to 15 min. In contrast, the re-organization of YFP-STIM1 induced by LPA appeared to be relatively transient, and puncta did not persist at the PM for as long as those generated by UTP stimulation. This is the first evidence of differential regulation of agonist-induced STIM1 redistribution and suggests that PLC-activating agonists can be classified into those that promote sustained localization of STIM1 puncta to the PM, and those for which puncta formation is relatively short-lived. Interestingly, even though PM levels of STIM1 were similar for UTP and LPA at 5 min (Fig. 4E), [Ca\(^{2+}\)]\(_{i}\) was significantly higher at that time point for

![Fig. 5. Differential store refilling after agonist-induced Ca\(^{2+}\) release. A: HaCaT keratinocytes loaded with Fluo-4 in nominally Ca\(^{2+}\)-free KHB were stimulated with 10 \(\mu\)M of UTP (open circles, \(n = 27\) cells) or LPA (filled squares, LPA \(n = 32\) cells) as indicated by the arrow. After the Ca\(^{2+}\) signal had declined to baseline, cells were stimulated with 1 \(\mu\)M TG (arrow head). Data are from one representative experiment, similar results were obtained in three independent experiments. B: The peaks of the agonist-induced or TG-induced Ca\(^{2+}\) signals were pooled from three independent experiments (\(n = 112\) cells in each case).]

![Fig. 6. Schematic representation of an agonist-induced Ca\(^{2+}\) influx network. Activation of G protein-coupled receptors (GPCR) by an agonist (A) stimulates phospholipase C-\(\beta\) (PLC-\(\beta\)) activity via G proteins (not shown). The diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\(_3\)) molecules subsequently generated by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) can activate Ca\(^{2+}\) entry through TRPC channels and cell surface IP\(_3\) receptors (IP\(_R\), respectively. Discharge of endoplasmic reticulum Ca\(^{2+}\) stores by IP\(_R\) appears to trigger Ca\(^{2+}\) influx via at least three distinct pathways: CIF-iPLA\(_2\), STIM1-Orai1, and STIM1-TRPC. See text for details. For clarity, the Ca\(^{2+}\)-calmodulin complex that CIF displaces from iPLA\(_2\) is not shown. CIF, Ca\(^{2+}\)-influx factor; iPLA\(_2\), Ca\(^{2+}\)-independent phospholipase A\(_2\); PL, phospholipids; LPL, lysophospholipids; TRPC, canonical transient receptor potential channel.]

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UTP (compare Figs. 2A and 3A). This difference suggests that other pathways activated by LPA may exert negative feedback on Ca\(^{2+}\) entry at the STIM1–Orai1 nexus. Several studies have shown that PLA\(_2\) activity is required for a range of intracellular trafficking events, such as retrograde membrane trafficking from the Golgi and trans-Golgi network (TGN) to the ER, and endocytic recycling of transferrin receptors (de Figueiredo et al., 2000, 2001). The redistribution of STIM1, however, appears to be independent of PLA\(_2\) activity as incubation of cells expressing YFP-STIM1 with BEL for 30 min did not has any discernable effect on the translocation of YFP-STIM1 (data not shown). This does not, however, imply that BEL treatment has no effect on STIM1-mediated Ca\(^{2+}\) entry. For instance, BEL (that is, inhibition of PLA\(_2\)) could potentially inhibit the fundamental mechanism, as yet unknown, by which STIM1 activates Orai1. Further investigations will be required to establish whether there is any crosstalk between PLA\(_2\) and STIM1-dependent Ca\(^{2+}\) entry. Why do the Ca\(^{2+}\) stores seem to be reloaded more rapidly after LPA stimulation compared to UTP stimulation? Significant amounts of the Ca\(^{2+}\) released from intracellular reservoirs are extruded from cells by the plasma membrane Ca\(^{2+}\)-ATPase (PMCA)(Parekh and Penner, 1997). Inhibition of PMCA would impair this extrusion, leaving more residual Ca\(^{2+}\) for recharging of the stores by SERCA pumps. Thus differential regulation of PMCA activity may explain the observed differences in store refilling after LPA and UTP stimulation. This may be mediated by differential production of H\(_2\)O\(_2\), a n
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