Lysophosphatidic Acid Promotes Cell Migration through STIM1- and Orai1-Mediated Ca$^{2+}$; Mobilization and NFAT2 Activation

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Lysophosphatidic acid (LPA) enhances cell migration and promotes wound healing in vivo, but the intracellular signaling pathways regulating these processes remain incompletely understood. Here we investigated the involvement of agonist-induced Ca$^{2+}$ entry and STIM1 and Orai1 proteins in regulating nuclear factor of activated T cell (NFAT) signaling and LPA-induced keratinocyte cell motility. As monitored by Fluo-4 imaging, stimulation with 10$\mu$M LPA in 60$\mu$M Ca$^{2+}$$_o$ evoked Ca$^{2+}$$_i$ transients owing to store release, whereas addition of LPA in physiological 1.2 mM Ca$^{2+}$$_o$ triggered store release coupled to extracellular Ca$^{2+}$ entry. Store-operated Ca$^{2+}$ entry (SOCE) was blocked by the SOCE inhibitor diethylstilbestrol (DES), STIM1 silencing using RNA interference (RNAi), and expression of dominant/negative Orai1R91W. LPA induced significant NFAT activation as monitored by nuclear translocation of green fluorescent protein-tagged NFAT2 and a luciferase reporter assay, which was impaired by DES, expression of Orai1R91W, and inhibition of calcineurin using cyclosporin A (CsA). By using chemotactic migration assays, LPA-induced cell motility was significantly impaired by STIM1, CsA, and NFAT2 knockdown using RNAi. These data indicate that in conditions relevant to epidermal wound healing, LPA induces SOCE and NFAT activation through Orai1 channels and promotes cell migration through a calcineurin/NFAT2-dependent pathway.

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INTRODUCTION

Lysophosphatidic acid (LPA) is a potent bioactive phospholipid that regulates diverse cellular processes including proliferation, differentiation, adhesion, and migration among several cell types, principally through interaction with a range of specific cell-surface receptors (Noguchi et al., 2009). In the context of wound repair, LPA is released into acute wounds from serum of LPA to its specific receptors also elicits mobilization of intracellular calcium (Ca$^{2+}$$_i$) from IP$_3$-dependent stores and influx of extracellular Ca$^{2+}$ + (Ca$^{2+}$$_o$) mediated by incompletely defined mechanisms (Noguchi et al., 2009). Keratinocytes are known to express LPA$_1$–3 and respond to LPA with Ca$^{2+}$ transients (Anliker and Chun, 2004; Roedding et al., 2006; Ross et al., 2007; Lichte et al., 2008), but the physiological relevance of LPA-induced Ca$^{2+}$ fluxes and the downstream signaling pathways in mediating specific physiological effects remain to be defined.

Recent studies have identified the endoplasmic reticulum Ca$^{2+}$ sensor STIM1 and the plasma membrane channel protein Orai1 as critical components of agonist-induced Ca$^{2+}$ entry (Frischauf et al., 2008; Martin et al., 2009), as well as being crucial for tumor cell migration (Vassilieva et al., 2008; Yang et al., 2009; Zuo and Chen, 2009). Our previous data suggested a role for STIM1 in mediating Ca$^{2+}$ entry in keratinocytes (Ross et al., 2007, 2008), but there is currently no information regarding the role of STIM1 or Orai1 in regulating keratinocyte migration.
The calcineurin/nuclear factor of activated T cell (NFAT) signaling cascade is characteristically recruited by the sustained Ca$^{2+}$i mobilization observed during Ca$^{2+}$ entry (Macian, 2005; Gwack et al., 2007). NFAT factors have been determined to be crucial for regulating cell motility in several cell types (Jauliac et al., 2002; Corral et al., 2007). We and others have shown that NFAT factors are functionally active in keratinocytes and epidermis, and calcineurin/NFAT activity has also been directly implicated in the regulation of keratinocyte growth and differentiation (Santini et al., 2001; Al-Daraji et al., 2002; Mammucari et al., 2005). The relationship between Ca$^{2+}$ fluxes and NFAT activation in keratinocytes remains to be determined, and furthermore, the involvement of calcineurin/NFAT signaling in LPA-induced cell motility remains unknown.

In this study, we therefore investigated the hypothesis that LPA promotes keratinocyte migration through Ca$^{2+}$i mobilization and examined the role of STIM1, Orai1 and calcineurin/NFAT recruitment within these physiological phenomena.

RESULTS

**Stimulation of keratinocytes with LPA in physiological Ca$^{2+}$o triggers Ca$^{2+}$ entry**

As previous studies in lymphocytes have indicated that sustained increases in Ca$^{2+}$i encountered during Ca$^{2+}$ entry (Gwack et al., 2007) are required for NFAT activation (Dolmetsch et al., 1997), we first determined the experimental conditions in which LPA could evoke Ca$^{2+}$ entry in primary keratinocytes. LPA is known to induce short transient elevations of Ca$^{2+}$i due to the release of Ca$^{2+}$ from intracellular stores, but Ca$^{2+}$i entry has not been reported (Ross et al., 2007, 2008; Lichte et al., 2008). As the relevance of Ca$^{2+}$o in the regulation of LPA-induced Ca$^{2+}$ mobilization in keratinocytes has largely been ignored, we investigated LPA-induced Ca$^{2+}$ mobilization in the context of varying Ca$^{2+}$o.

Keratinocytes were routinely cultured in medium containing 60 μM Ca$^{2+}$o. The addition of 10 μM LPA in these conditions induces a transient (± 200 seconds) peak elevation of Ca$^{2+}$i (Figure 1a), indicative of store release as expected (Ross et al., 2007, 2008; Lichte et al., 2008). Adjustment of the Ca$^{2+}$o to 1.2 mM in the absence of LPA resulted in a weak increase in Ca$^{2+}$i (Figure 1a) over an extended period of time (>10 minutes), similar to previous reports (Bikle et al., 1996; Tu et al., 2001). To mimic the contact of keratinocytes with LPA in a serum-relevant Ca$^{2+}$o, we analyzed the effect of adding LPA in 1.2 mM Ca$^{2+}$o. Under these conditions, LPA induced a transient peak in Ca$^{2+}$i followed by a sustained (>10 minutes) plateau elevation of Ca$^{2+}$i, which suggests store release coupled to Ca$^{2+}$ entry (Figure 1a).

We then used an addback protocol to verify the occurrence of Ca$^{2+}$ entry by uncoupling store release from influx (Figure 1b). Manganese quenching (Sage et al., 1989) by the addition of 500 μM MnCl2 during the plateau phase quickly reduced Ca$^{2+}$i to the baseline level (Figure 1b). In addition, we used Ca$^{2+}$ entry–blocking pharmacological agents to further define the Ca$^{2+}$-mobilizing effect of LPA. We initially validated the pharmacological agents through studies on thapsigargin (Tg)-induced Ca$^{2+}$ entry. Although 2-aminoethoxydiphenyl borate has been classically used to block store-operated Ca$^{2+}$ entry (SOCE), this molecule is increasingly recognized to be rather nonspecific (Schindl et al., 2008). We found that 2-aminoethoxydiphenyl borate did not block Tg-induced Ca$^{2+}$ entry at a variety of concentrations (data not shown). Similarly, we found that the addition of gadolinium ions (Gd3+) did not reliably block Tg-induced SOCE (data not shown). We therefore used the lesser known SOCE inhibitor diethylstilbestrol (DES), which blocks SOCE channels and inwardly rectifying current but exerts no effect on TRPM channels (Zakharev et al., 2004; Dobrydneva et al., 2010). DES was indeed found to inhibit Tg-induced SOCE in keratinocytes (Figure 1c), and the addition of DES during the plateau phase of LPA-induced Ca$^{2+}$ entry blocked further entry (Figure 1b), providing further evidence that LPA induces Ca$^{2+}$ entry in keratinocytes through SOCE channels.

To further characterize the impact of Ca$^{2+}$o on LPA-induced Ca$^{2+}$ mobilization, keratinocytes were incubated in medium containing 1.2 mM Ca$^{2+}$ for an extended time (45 minutes) before LPA stimulation. Under these conditions, LPA induced store release (Figure 1d) in a manner similar to previous studies (Ross et al., 2007; Lichte et al., 2008), but no entry was apparent during the observed time span (>10 minutes). Next, we uncoupled the addition of agonist and Ca$^{2+}$ by adjusting the Ca$^{2+}$o to 1.2 mM for 60 seconds before adding LPA (Roedding et al., 2006) (Figure 1e). There was no significant difference detected between Ca$^{2+}$i fluxes observed in these conditions and when LPA and millimolar Ca$^{2+}$ were added simultaneously, suggesting that simultaneous stimulation of LPA receptors and adjustment to millimolar Ca$^{2+}$ does not just overwhelm the plasma membrane channels and thereby cause nonspecific entry.

**LPA induces nuclear translocation of NFAT2 and upregulates NFAT transcriptional activity**

We then investigated the effect of LPA on NFAT activation. First, we used a real-time live-cell imaging method to simultaneously analyze Ca$^{2+}$ variations (using FuraRed) and NFAT activation (by visualizing nuclear translocation of green fluorescent protein (GFP)-tagged NFAT2). The proof-of-principle of this method was achieved by triggering Tg-mediated SOCE, which resulted in a sustained Ca$^{2+}$i increase as expected. This correlated with progressive and strong nuclear accumulation of NFAT2-GFP within 10 minutes of exposure (Figure 2a).

Simultaneous stimulation with LPA and 1.2 mM Ca$^{2+}$ resulted, as expected, in Ca$^{2+}$ store release coupled to entry, which correlated with progressively increasing nuclear accumulation of NFAT2 (Figure 2b). Adjustment of Ca$^{2+}$o to 1.2 mM did not induce significant NFAT2 translocation within 10 minutes of treatment (Figure 2c). In all experimental conditions, nuclear translocation of NFAT2 was prevented by preincubating the cells for 1 hour with the calcineurin inhibitor cyclosporin A (CsA, 1 μM) before agonist challenge (data not shown).

Second, we investigated the effect of LPA on NFAT activity using a luciferase reporter under the transcriptional control of
NFAT (Wilkins et al., 2004). LPA stimulation in 1.2 mM Ca\(^{2+}\)\(_o\) prompted a significant and sustained upregulation of NFAT activity to a level ~10-fold over baseline within 4 hours after stimulation (Figure 3a). In comparison, LPA stimulation in 60 \(\mu\)M Ca\(^{2+}\)\(_o\) increased NFAT activity within 4 hours before reaching a plateau level ~2.5-fold over the control level (Figure 3a). Adjustment of Ca\(^{2+}\)\(_o\) to 1.2 mM in the absence of LPA induced a weak increase of NFAT activity (~2.5-fold) over 24 hours (Figure 3a). As shown in Figure 3b, preincubation of keratinocytes for 1 hour with 1 \(\mu\)M CsA before LPA and millimolar Ca\(^{2+}\)\(_o\) challenge prevented the induction of NFAT transcriptional activity. To further investigate the role of Ca\(^{2+}\) entry in LPA-induced NFAT activation, DES was added during agonist challenge, which resulted in a significantly impaired induction of NFAT activity (Figure 3c).

Characterization of the mechanism of LPA-induced Ca\(^{2+}\) entry

The implication of STIM1 in LPA-induced Ca\(^{2+}\) entry was studied by analyzing the effect of RNA interference (RNAi-mediated) knockdown of STIM1 expression using nucleofection, which achieves efficient transfection of primary keratinocytes (Supplementary Figure S1 online). Figure 4a-c illustrates that keratinocytes transfected with small interfering RNA (siRNA) directed against STIM1 exhibit a significant decrease in STIM1 mRNA and protein content 24 hours post transfection. Figure 4d shows that LPA-induced Ca\(^{2+}\) entry was impaired in STIM1 siRNA-transfected keratinocytes compared with scrambled siRNA-transfected cells.

The involvement of Orai1 in LPA-induced Ca\(^{2+}\) influx was investigated by evaluating the impact of overexpressing Orai1\(^{R91W}\), an Orai1 mutant that substantially attenuates Ca\(^{2+}\) entry through Orai1 channels through a dominant-negative effect (Liao et al., 2007). Figure 4e and f illustrates efficient transfection of Orai1-encoding plasmids to keratinocytes by nucleofection. As shown in Figure 4g, LPA-induced Ca\(^{2+}\) influx was inhibited in Orai1\(^{R91W}\)-expressing keratinocytes, whereas entry levels were comparable in wild-type Orai1-expressing and empty vector–transfected cells. Notably, subsequent LPA-induced upregulation of NFAT transcriptional activity was found to be impaired in Orai1\(^{R91W}\)-expressing cells, but not in wild-type Orai1- or empty vector–transfected cells (Figure 4h). A similar effect was observed following siRNA-mediated knockdown of Orai1 (Supplementary Figure S2 online).

LPA-induced keratinocyte migration requires STIM1, calcineurin, and NFAT2

To test the involvement of STIM1 in mediating LPA-induced keratinocyte migration, keratinocytes were subjected to STIM1 knockdown before performing three-dimensional (3D) chemotactic migration assays. Cells were allowed to migrate over 14 hours in medium containing 60 \(\mu\)M Ca\(^{2+}\)\(_o\) or 1.2 mM Ca\(^{2+}\) to mimic the contact of keratinocytes with LPA in high Ca\(^{2+}\)\(_o\), as would occur following wounding of skin. As described previously, exposure to millimolar Ca\(^{2+}\)\(_o\) dampened overall migration rates (Figure 5a and b) (Magee et al., 1987). In both Ca\(^{2+}\)\(_o\) conditions, LPA treatment induced a significant increase in keratinocyte motility, as expected (Sauer et al.,
However, LPA-induced migration was significantly impaired in the 3D assays (Figure 5b) following STIM1 knockdown, suggesting that normal levels of STIM1 are required for LPA-induced keratinocyte motility.

To investigate the involvement of the calcineurin/NFAT pathway in mediating LPA-induced migration, cells were pretreated using the calcineurin inhibitor CsA before being subjected to two-dimensional scratch wounding (Supplementary Figure S3 online) and 3D chemotactic migration assays (Figure 5c and d). LPA-induced migration was significantly impaired in CsA-treated keratinocytes. Moreover, RNAi-mediated knockdown of NFAT2, the efficiency of which was verified by real-time PCR (Figure 5e) and western blotting (Figure 5f and g), significantly prevented the induction of 3D migration of keratinocytes by LPA in 60 μM and 1.2 mM Ca\(^{2+}\) (Figure 5h and i).

**DISCUSSION**

The data presented in this paper (schematically summarized in Figure 6) demonstrate that stimulation of keratinocytes with LPA induces Ca\(^{2+}\)\(\text{_{o}}\)-regulated mobilization of Ca\(^{2+}\), leading to the activation of calcineurin/NFAT2 signaling and keratinocyte migration. LPA stimulation in physiological millimolar Ca\(^{2+}\)\(\text{_{o}}\) evoked agonist-induced Ca\(^{2+}\) entry, which required sufficient levels of STIM1 and functional Orai1. Downstream of LPA-induced Ca\(^{2+}\) mobilization, we observed nuclear...
Figure 3. Lysophosphatic acid (LPA)-induced Ca\(^{2+}\) entry triggers sustained calcineurin-dependent upregulation of nuclear factor of activated T cell (NFAT) transcriptional activity. (a) Keratinocytes were stimulated as indicated and assessed for NFAT-dependent luciferase activity expressed as mean ± SEM. (b, c) Calcineurin activity (b) and Ca\(^{2+}\) entry (c) were required for LPA-induced NFAT activation (*P<0.05, **P<0.01, one-way analysis of variance).

Figure 4. Lysophosphatic acid (LPA)-induced Ca\(^{2+}\) entry requires sufficient levels of STIM1 and functional Orai1. (a–c) Relative efficiency of small interfering RNA (siRNA)-mediated STIM1 silencing compared to scrambled (scr) control by quantitative PCR and western blotting. (e) Fura-PE3-based Ca\(^{2+}\) imaging showed impaired LPA-induced Ca\(^{2+}\) entry in STIM1-knockdown cells. (e, f) Efficient transfection of Orai1-encoding plasmids demonstrated by western blotting for myc-tag. (g) LPA-induced Ca\(^{2+}\) entry was found to be blocked in Orai1\(^{R91W}\)-expressing cells. (h) Keratinocytes were cotransfected with Orai1, nuclear factor of activated T cell (NFAT)-directed, and Renilla luciferase plasmids. Cells were then treated with LPA and 1.2 mM Ca\(^{2+}\) and assessed for NFAT transcriptional activity. Overexpression of Orai1\(^{R91W}\) significantly impaired NFAT activation. Data represent mean ± SEM; n=3 unless otherwise stated, *P<0.05, **P<0.01, ***P<0.001, analysis of variance.
Figure 5. Lysophosphatidic acid (LPA)-induced keratinocyte migration requires STIM1, calcineurin activity, and sufficient levels of nuclear factor of activated T cell 2 (NFAT2). (a–d, h, i) Keratinocyte cultures were subjected to three-dimensional (3D) chemotactic migration assays, which were performed in medium containing 60 μM or 1.2 mM Ca²⁺. The average numbers of migrated cells are represented as bar graphs ± SEM. Keratinocytes were treated with scrambled (scr) or STIM1-targeted small interfering RNA (siRNA) (a, b) or NFAT2-targeted siRNA (h, i) for 24 hours prior to 3D chemotactic migration assays. In both Ca²⁺ conditions, 10 μM LPA significantly increased migration rates. RNA interference (RNAi)-mediated knockdown of STIM1 and NFAT2 resulted in a significant impairment of LPA-induced keratinocyte motility (a, b, h, i, ***P < 0.001, **P < 0.01, two-way analysis of variance (ANOVA)). (c, d) Preincubation of cells with 1 μM cyclosporin A significantly impaired LPA-induced migration (n ≥ 3, **P < 0.01, *P < 0.05, two-way ANOVA). (e–g) Keratinocytes were transfected using scr or NFAT2-targeted siRNA. After 24 hours, cells were subjected to (e) real-time PCR analysis of NFAT2 expression (n = 6), (f) western blot analysis, and (g) densitometry (n = 3). RNAi-mediated knockdown of NFAT2 is significant (*P < 0.05, t-test). Bar = 50 μm.
translocation of NFAT2 and activation of NFAT-dependent transcription. Finally, LPA-induced keratinocyte motility required sufficient levels of STIM1 and NFAT2 and calcineurin activity. Our study thus provides important insight into the signaling mechanism triggered by LPA to promote keratinocyte migration.

The physiology of keratinocytes is well known to be tightly regulated by Ca\textsuperscript{2+}\textsubscript{o} (Tu et al., 2004). Consistent with this, our data show differential Ca\textsuperscript{2+}\textsubscript{o} mobilization and NFAT activation upon stimulation with LPA in physiological millimolar Ca\textsuperscript{2+}\textsubscript{o} compared with micromolar levels. Indeed, LPA induced Ca\textsuperscript{2+}\textsubscript{o} entry when the Ca\textsuperscript{2+}\textsubscript{o} was adjusted to 1.2 mM simultaneously with or briefly before stimulation. However, keratinocytes left in 1.2 mM Ca\textsuperscript{2+}\textsubscript{o} for a prolonged time before LPA stimulation only exhibited store release, but no Ca\textsuperscript{2+}\textsubscript{o} entry was detectable within the observed time span, which is in accordance with previous studies (Ross et al., 2007, 2008; Lichte et al., 2008) and suggests that prolonged exposure to millimolar Ca\textsuperscript{2+} results in the inhibition of acute LPA-induced Ca\textsuperscript{2+} entry. Our data indicating NFAT activation caused by Ca\textsuperscript{2+} entry are consistent with previous studies performed in other epithelial cell types, including prostate (Thebault et al., 2006) and kidney (Schlondorff et al., 2009) cells.

The role of store- and receptor-operated Ca\textsuperscript{2+} entry (SOCE/ROCE) during physiological activation of primary cells has not been extensively investigated, and thus there is little information on the roles of STIM and Orai proteins in primary cells except in hepatocytes, in which STIM1 and Orai1 are involved in mediating Ca\textsuperscript{2+} entry leading to agonist-induced oscillations (Jones et al., 2008). To our knowledge, the establishment of functional roles for STIM1 and Orai1 in primary keratinocytes as mediators of agonist-induced Ca\textsuperscript{2+} entry has previously been unreported. Our data showing that LPA-induced Ca\textsuperscript{2+} entry depends on normal levels of STIM1 and functional Orai1 are consistent with studies in which STIM1 knockdown (Hirano et al., 2009; Potier et al., 2009) and expression of dominant-negative Orai1\textsuperscript{R91W} (Liao et al., 2007, 2008) lead to the inhibition of SOCE or ROCE in a variety of cell lines.

Our data concur with an increasing body of evidence indicating an emerging role for Ca\textsuperscript{2+} signaling as a critical regulator of cell motility in a variety of physiological and pathological contexts. For instance, STIM1 and Orai1 have been found to mediate agonist-induced Ca\textsuperscript{2+} entry and regulate Ca\textsuperscript{2+} flux-dependent cell migration in epithelial tumor cells (Yang et al., 2009) and vascular smooth muscle cells (Potier et al., 2009). In addition, functional inhibition of calcineurin and NFAT2 impaired the migration rate of epithelial tumor cells (Jauliac et al., 2002), and wound closure in scratched myocyte cultures was reduced by silencing NFAT2 expression (Chow et al., 2008).

NFAT transcription factors have been previously attributed to important roles in the control of keratinocyte growth and differentiation (Santini et al., 2001; Mammucari et al., 2005; Horsley et al., 2008) and in the response of keratinocytes to UV-mediated stress (Canning et al., 2006; Flockhart et al., 2008). In conclusion, our work underscores the importance of Ca\textsuperscript{2+} signaling and NFAT factors in the control of epidermal physiology and potentially in cutaneous wound healing.

MATERIALS AND METHODS

Reagents

All chemicals were purchased from Sigma-Aldrich (Gillingham, UK) unless otherwise specified.

Cell culture and treatments

Normal human epidermal keratinocytes were isolated from redundant foreskins (Todd and Reynolds, 1998) or breast or abdominal skin (Jans et al., 2004), and primary cultures were expanded in human keratinocyte growth supplement Epilife medium (Invitrogen, Paisley, UK). The study adhered to the Declaration of Helsinki protocols for use of human materials, was approved by the Newcastle and North Tyneside local ethics committee, and written informed patient consent was obtained. Keratinocytes were used between passage 2 and 5. Treatments with agonists were performed by diluting the agonists directly in medium. For inhibition of calcineurin activity, cells were pretreated with 1\mu M CsA (Calbiochem, San Diego, CA) for 1 hour before agonist challenge. The concentration of LPA used throughout the study was 10\mu M, which is a generally accepted concentration (Ross et al., 2007, 2008; Lichte et al., 2008).

Ca\textsuperscript{2+} imaging

Keratinocytes seeded in Willco glass-bottomed dishes (Intracel, Royston, UK) were subjected to Ca\textsuperscript{2+}; imaging using Fluo-4-AM (Invitrogen) or Fura-PE3-AM (Calbiochem) as described (Ross et al., 2007). Changes in Ca\textsuperscript{2+}; were monitored at 10-second intervals with a Pathway HTS imaging system (Becton-Dickinson, Rockville, MD).
Images were captured using an Olympus (Melville, NY) ×20 objective and a Hamamatsu 1394 (Hamamatsu, Japan) ORCA-ERA CCD camera. Fura-2 was excited at 488 nm, collecting emitted fluorescence through a 515 LP filter. Fura-PE3 was excited at 340 and 380 nm, and emitted fluorescence was collected through the 515 LP filter. Quantification was performed using Volocity (Improvision, Coventry, UK). When using Fluor-4, changes in Ca²⁺; were expressed as the ratio of the initial fluorescence to the temporal fluorescence (Fᵢ/F₀). When using Fura-PE3, changes in Ca²⁺; were expressed as ratiometric measurements (F₃₄₀nm/F₃₈₀nm).

Dual imaging of Ca²⁺; and NFAT2-GFP
Keratinocytes transduced with NFAT2-GFP-delivering retroviruses (Flockhart et al., 2008) were seeded on Willco dishes. After 24 hours, the cells were loaded using 5 μM FuraRed-AM (Invitrogen). Dual imaging was then performed using a Leica TCS SP2 confocal laser scanning microscope equipped with an argon laser (Leica, Milton Keynes, UK). Fluorescence excitation of GFP and FuraRed was performed with the 488 nm line of the laser. Fluorescence emission of GFP was collected through a 500–550-nm window of the detector, whereas FuraRed emission was collected through a 650–700-nm window. Images were captured with a ×63 Plan Apo objective (NA1.32) (Leica, Milton Keynes, UK) at 10-second intervals. Changes in Ca²⁺; are expressed as the inverted ratio of the initial fluorescence to the temporal fluorescence (1/Fᵢ/F₀). The nuclear-to-cytosplasmic ratio of NFAT2-GFP was assessed by outlining the nucleus and cytoplasm of each cell as separate regions of interest, calculating the mean green fluorescence in each region of interest and frame in Volocity. Nuclear-to-cytosplasmic ratios were then calculated in Microsoft Excel.

NFAT transcriptional activity assay
NFAT transcriptional activity was measured using an NFAT firefly luciferase reporter plasmid (pGL3; Promega, Southampton, UK), containing nine copies of an NFAT-binding site from the IL-2 promoter (5'-TGGAATATT-3') positioned 5' to a minimal promoter from the β-myosin heavy-chain gene, gratefully obtained from J. Molkentin, Cincinnati, OH (Wilkins et al., 2004). Transfection efficiency and cell viability were controlled by cotransfecting a Renilla luciferase control vector (pRLTK; Promega). Keratinocytes were seeded in 12-well plates and transfected using 0.5 μg of firefly reporter DNA plus 0.05 μg of Renilla luciferase DNA using Fugene 6 (Roche Applied Sciences, Burgess Hill, UK) at a 6:1 Fugene:DNA ratio. After 24 hours, the cells were stimulated as described in Sauer et al., 2004. Briefly, 20,000 keratinocytes were seeded on top of uncoated Transwell filters (12 mm diameter, 8 μm pore size; Corning Life Sciences, Schiphol-Rijk, The Netherlands) in a 10-μl drop. Care was taken to deposit the drop in the middle of the filter without it touching the plastic support walls, in order to prevent a meniscus effect causing unequal distribution. The cells were left to attach for 30 minutes at room temperature before adding 300 μl of EpLife medium containing agonists into the lower compartment. The cells were left to migrate overnight at 37 °C before fixation using 4% formaldehyde. Unmigrated cells were scraped off the top portion of the filters using cotton buds. The filters were stained using hematoxylin and eosin, cut out and mounted using DPX. The migrated cells were counted on a Leica photomicroscope using a ×10 objective (3–5 fields per sample).

Effect of dominant/negative Orai1R91W on LPA-induced Ca²⁺ entry
pCMV-myc plasmids encoding wild-type Orai1 or mutated dominant/negative Orai1R91W were kindly donated by Professor L Birnbaumer (Liao et al., 2007) (NIEHS, Research Triangle Park, NC). Keratinocytes (10⁶ cells) were transfected by nucleofection with 2 μg of Orai1-encoding plasmid and 0.5 μg of pEGFP-N1 (Clontech, Mountain View, CA) to mark the transfected cells (Zaraysky et al., 2007). The expression of myc-tagged Orai1-encoding plasmids was verified by standard western blotting using anti-myc antibody (clone 9E10; Santa Cruz Biotechnology, San Diego, CA). Cells were seeded into Willco dishes at 0.5 × 10⁶ cells per dish. After 24 hours, the cells were processed for Ca²⁺ imaging using Fura-PE3. Before performing Ca²⁺ imaging, the sample field was analyzed for GFP expression using the appropriate filter sets.

Migration assays
Keratinocyte motility was assessed using standard two-dimensional scratch wounding and 3D chemotactic migration assays. Chemotactic migration of keratinocytes was assayed in a manner similar to that described in Sauer et al., 2004. Briefly, 20,000 keratinocytes were seeded on top of uncoated Transwell filters (12 mm diameter, 8 μm pore size; Corning Life Sciences, Schiphoul-Rijk, The Netherlands) in a 10-μl drop. Care was taken to deposit the drop in the middle of the filter without it touching the plastic support walls, in order to prevent a meniscus effect causing unequal distribution. The cells were left to attach for 30 minutes at room temperature before adding 300 μl of EpLife medium containing agonists into the lower compartment. The cells were left to migrate overnight at 37 °C before fixation using 4% formaldehyde. Unmigrated cells were scraped off the top portion of the filters using cotton buds. The filters were stained using hematoxylin and eosin, cut out and mounted using DPX. The migrated cells were counted on a Leica photomicroscope using a ×10 objective (3–5 fields per sample).
Statistical analysis
Statistical analysis was performed using Prism 5 (GraphPad Software, San Diego, CA). Data represent mean ± SEM. Experiments were repeated at least three times using different keratinocyte strains unless otherwise specified.

CONFLICT OF INTEREST
The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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