Lysophospholipid Receptor-Mediated Calcium Signaling in Human Keratinocytes

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The lysophospholipids, sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA), stimulate chemotaxis and induce differentiation of human keratinocytes. As Ca^{2+} plays an important role in keratinocyte differentiation, we studied Ca^{2+} signaling by S1P and LPA in these cells, known to express mRNA transcripts of the $S1P_{1-5}$ and LPA_{1-3} receptors, and the receptor subtypes involved in this process. S1P and LPA caused transient increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), with pEC_{50} values of 8.5 ± 0.11 and 7.5 ± 0.23, respectively. The $[Ca^{2+}]_i$ increases are apparently mediated by stimulation of phospholipase C and involve Ca^{2+} mobilization from thapsigargin-sensitive stores and subsequent Ca^{2+} influx. The LPA-induced $[Ca^{2+}]_i$ increases were not inhibited by the $LPA_{1/3}$ receptor antagonist, dioctanoylglycerol pyrophosphate. The S1P-induced $[Ca^{2+}]_i$ increases were largely inhibited by the putative $S1P_3$ antagonist, BML-241, and the $S1P_{1/3}$ antagonist, VPC23019. The $S1P_1$-specific agonist, SEW2871, did not increase $[Ca^{2+}]_i$ but stimulated chemotaxis of keratinocytes, which was fully blocked by $S1P_1$ antisense oligonucleotides. The data indicate that LPA and S1P potently increase $[Ca^{2+}]_i$ in human keratinocytes and that the effect of LPA is mediated by LPA_2, whereas that of S1P is mediated at least to a large part by $S1P_3$. The $S1P_1$ receptor, without stimulating $[Ca^{2+}]_i$ increases, mediates chemotaxis of keratinocytes.

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INTRODUCTION

The lysophospholipids, sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA), are ubiquitous autocrine and paracrine mediators regulating cell growth and survival, differentiation, cell motility, adhesion, and intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in many cell types (for reviews, see Spiegel and Milstien, 2003; Tigyi and Parrill, 2003; Anliker and Chun, 2004; Sanchez and Hla, 2004; Rosen and Goetzl, 2005; Gardell *et al.*, 2006). S1P plays a decisive role for vascular maturation and regulates angiogenesis, vascular tone and permeability, heart rate, trafficking of lymphocytes, and tumour cell growth (Spiegel and Milstien, 2003; Anliker and Chun, 2004; Sanchez and Hla, 2004; Rosen and Goetzl, 2005; Visentin *et al.*, 2006). LPA regulates implantation, contributes to the development of the nervous system, has an antiadipogenic activity, and plays a role in inflammation and arteriosclerosis (Tigyi and Parrill, 2003; Anliker and Chun, 2004; Siess and Tigyi, 2004; Simon *et al.*, 2005; Ye *et al.*, 2005). Other physiological or pathophysiological roles for lysophospholipids have been suggested. The above-mentioned actions of S1P and LPA are mediated by a network of specific G protein-coupled receptors, of which the receptors of the endothelial differentiation gene family, $S1P_{1-5}$ and LPA_{1-3}, have been characterized in greatest detail (Tigyi and Parrill, 2003; Anliker and Chun, 2004; Sanchez and Hla, 2004; Rosen and Goetzl, 2005). Two other, more distantly related G protein-coupled LPA receptors and three more putative S1P receptors have furthermore been identified (Uhlenbrock *et al.*, 2002; Noguchi *et al.*, 2003; Lee *et al.*, 2006). In addition, LPA acts intracellularly as a weak activator of the ligand-activated transcription factor, peroxisome proliferator-activated receptor-γ, whereby it promotes neointima formation (McIntyre *et al.*, 2003; Zhang *et al.*, 2004). S1P, independently of its role as agonist at G protein-coupled receptors, also functions as intracellular second messenger and thereby transduces signals of diverse receptors; however, its intracellular target sites have not been identified so far (reviewed in Spiegel and Milstien, 2003; Meyer zu Heringdorf, 2004).

Sphingolipids have long been recognized as important structural components of the epidermis, securing the epidermal permeability barrier. Moreover, recent findings suggest a substantial role of S1P and LPA as bioactive molecules in keratinocytes involved in the regulation of cell growth and differentiation. Although S1P and LPA are potent mitogens in a variety of cells, an opposite effect is apparent in

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Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CFP, cyan fluorescent protein; DGPP 8:0, dioctanoylglycerol pyrophosphate; GFP, green fluorescent protein; IP_3, inositol-1,4,5-trisphosphate; LPA, lysophosphatidic acid; PLC, phospholipase C; S1P, sphingosine-1-phosphate; SPC, sphingosylphosphorylcholine

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human keratinocytes, as both mediators inhibit growth of the epidermal cells. However, S1P and LPA do not drive keratinocytes into apoptosis but rather induce their differentiation as assessed by transglutaminase activity (Manggau *et al.*, 2001; Vogler *et al.*, 2003; Sauer *et al.*, 2004b). S1P and LPA also induce migration of keratinocytes to denuded areas of the culture dish, implying a role of these lipids in the re-epithelialization of wounds.

It is well established that the extracellular Ca^{2+} concentration is an important regulator of keratinocyte differentiation *in vitro* (Hennings *et al.*, 1980; Watt *et al.*, 1984). An early response to an increase in the extracellular Ca^{2+} concentration is an increase in $[Ca^{2+}]_i$ (Hennings *et al.*, 1989; Pillai *et al.*, 1990). Most interestingly, blocking the rise in $[Ca^{2+}]_i$ with intracellular Ca^{2+} chelators inhibits the differentiation process, suggesting that $[Ca^{2+}]_i$ increases are important in initiating the differentiation of keratinocytes (Li *et al.*, 1995). As S1P and LPA are potent inductors of keratinocyte differentiation, it was of great interest to further elucidate the role of the lipid mediators on Ca^{2+} signaling in these cells. Moreover, the first lysophospholipid receptor subtype-specific compounds have become available recently (Parrill *et al.*, 2004). Therefore, we studied Ca^{2+} signaling by lysophospholipids in human keratinocytes and analyzed the involved lysophospholipid receptor subtypes. In addition, we compared the effects of the receptor subtype-specific compounds in human keratinocytes to those in HEK-293 (human embryonic kidney) cells, as these cells have been used before for the analysis of lysophospholipid-mediated Ca^{2+} signaling (van Koppen *et al.*, 1996; Meyer zu Heringdorf *et al.*, 2001, 2003b).

RESULTS AND DISCUSSION

In agreement with previous reports demonstrating that S1P and LPA induce keratinocyte differentiation, the two lysophospholipids increased mRNA levels of the differentiation markers, keratin 1, keratin 10, and involucrin (Table 1). Both S1P and LPA caused transient $[Ca^{2+}]_i$ increases in adherent human keratinocytes loaded with fluo-4 (Figure 1). In concentrations maximal for activation of G protein-coupled lysophospholipid receptors, S1P and LPA caused rapid $[Ca^{2+}]_i$ peaks followed by a more sustained phase of $[Ca^{2+}]_i$ elevation, which slightly differed in shape between groups of cells and lasted 1–3 minutes (Figures 1a, b, and e, Figures 3 and 4). Concentrations of S1P just above threshold caused $[Ca^{2+}]_i$ increases with a variable delay, some cells did not respond (Figures 1d and g). A perinuclear vesicular compartment in keratinocytes did not accumulate fluo-4 (Figures 1f and g), which, together with confocal microscopy, allowed to easily differentiate between $[Ca^{2+}]_i$ increases in nuclei and cytoplasm. In most cells, basal fluo-4 fluorescence was slightly higher in the nucleus than in the cytosol and increased to a higher extent after stimulation, although any changes in $[Ca^{2+}]_i$ in the cytosol were paralleled by the same changes in the nucleus (Figure 1c).

For determination of concentration–response relationships of lysophospholipid-induced $[Ca^{2+}]_i$ increases, we used detached cells that could be measured in suspension with the ratioable compound, fura-2 (Figure 2). S1P and LPA increased $[Ca^{2+}]_i$ with pEC_{50} values of 8.5 ± 0.11 (Hill slope 0.47 ± 0.05; mean ± SEM; $n = 4$–5) and 7.5 ± 0.23 (Hill slope 0.51 ± 0.13; mean ± SEM; $n = 3$), respectively. The S1P-related lysophospholipid, sphingosylphosphorylcholine (SPC), increased $[Ca^{2+}]_i$ with pEC_{50} of 5.8 ± 0.03 (Hill slope 1.44 ± 0.12; mean ± SEM; $n = 3$). Maximum $[Ca^{2+}]_i$ increases by S1P, LPA, and SPC in suspended cells were 252 ± 33 nM (mean ± SEM; $n = 13$), 401 ± 30 nM (mean ± SEM; $n = 4$), and 291 ± 49 nM (mean ± SEM; $n = 4$), respectively. The EC_{50} values for $[Ca^{2+}]_i$ increases by S1P and SPC in keratinocytes are in the same range as in other cell types, for example, in HEK-293 and vascular endothelial cells (Meyer zu Heringdorf *et al.*, 1996; van Koppen *et al.*, 1996), with S1P being two to three orders of magnitude more potent than SPC. These data suggest that, in keratinocytes, SPC increases $[Ca^{2+}]_i$ by acting as a low-affinity agonist at S1P receptors, as it has been shown for recombinant $S1P_1$, $S1P_2$, and $S1P_3$ receptors (reviewed in Meyer zu Heringdorf *et al.*, 2002), rather than by activating a high-affinity SPC receptor such as OGR-1, which mediates $[Ca^{2+}]_i$ increases by SPC in the nanomolar range (Xu *et al.*, 2000). It is of particular interest that keratinocytes respond to SPC, as this lipid has been shown to accumulate in the stratum corneum in patients with atopic dermatitis (Okamoto *et al.*, 2003).

Table 1. mRNA levels of differentiation markers in human primary keratinocytes in response to 1 μM S1P and 1 μM LPA relative to cyclophilin A

	S1P	LPA
Keratin 1	108.6 ± 29.3	122.0 ± 22.4
Keratin 10	31.6 ± 1.7	85.7 ± 7.3
Involucrin	11.4 ± 1.2	8.2 ± 1.9

$[Ca^{2+}]_i$ increases by S1P were caused by mobilization of Ca^{2+} from intracellular stores and concomitant Ca^{2+} influx (Figure 3). In suspended cells, peak $[Ca^{2+}]_i$ increases by 1 μM S1P in the absence of extracellular Ca^{2+} reached $33.3 \pm 6.4\%$ of those in the presence of 1 mM extracellular Ca^{2+} (mean ± SEM; $n = 4$; Figure 3a). After incubation of keratinocytes in Ca^{2+}-free medium, the re-addition of 1 mM Ca^{2+} increased $[Ca^{2+}]_i$ by 248 ± 45 nM (mean ± SEM; $n = 3$). When the cells had been challenged with 1 μM S1P in Ca^{2+}-free medium, the re-addition of 1 mM Ca^{2+} increased $[Ca^{2+}]_i$ by 403 ± 66 nM (mean ± SEM; $n = 3$), and thus Ca^{2+} influx by S1P was 63% higher than Ca^{2+} influx caused by incubation in Ca^{2+}-free medium alone (Figure 3a). In adherent cells, peak $[Ca^{2+}]_i$ increases were not much diminished in the absence of extracellular Ca^{2+}, indicating that the overall $[Ca^{2+}]_i$ increase induced by S1P in adherent cells was largely due to Ca^{2+} mobilization (Figure 3b); however, elevated $[Ca^{2+}]_i$ returned back to baseline values much faster in the absence of extracellular Ca^{2+}.

In keratinocytes, the intracellular Ca^{2+} compartments of endoplasmic reticulum and Golgi apparatus seem to function

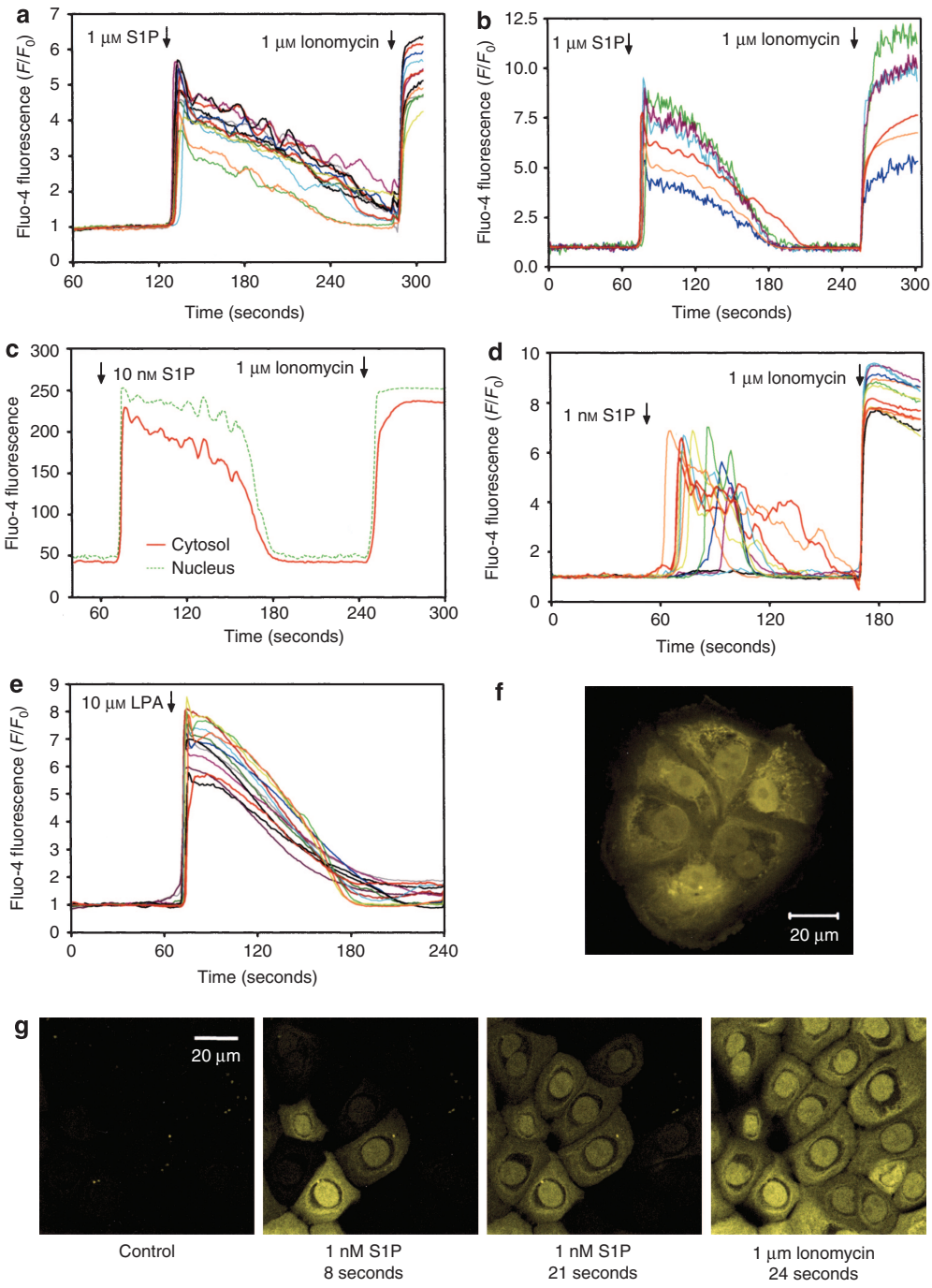

Figure 1. $[Ca^{2+}]_i$ increases by S1P and LPA in adherent keratinocytes. Keratinocytes grown on glass coverslips were loaded with fluo-4 and analyzed by confocal fluorescence microscopy. (**a**–**e**) The mean fluorescence in defined regions of interest, each line representing a single cell and each graph representing a group of cells measured in parallel. The cells were challenged with the indicated concentrations of S1P, LPA, and ionomycin. In (**c**), fluo-4 fluorescence was measured separately in the nucleus and the cytosol of one cell. Note that, in (**c**), for better comparison, fluorescence within the two compartments was not normalized to basal fluorescence. (**f**) High-resolution fluorescence image of a group of fluo-4-loaded keratinocytes. (**g**) Fluorescence images taken from a typical measurement, before (control) and at the indicated times after addition of 1 nM S1P or 1 μM ionomycin.

relatively separately, as mutations in sarco-endoplasmic reticulum Ca^{2+}-ATPase-2, a Ca^{2+} pump of endoplasmic reticulum, and secretory pathway Ca^{2+}-ATPase-1, pumping Ca^{2+} into the Golgi apparatus, cause the different phenotypes of Darier's and Hailey–Hailey disease, respectively (Behne *et al.*, 2003; Dhitavat *et al.*, 2004). Furthermore, the secretory pathway Ca^{2+}-ATPase-1-dependent Ca^{2+} stores in keratinocytes are of functional importance for agonist (ATP)-induced $[Ca^{2+}]_i$ increases (Callewaert *et al.*, 2003). Therefore, we studied the influence of the sarco-endoplasmic

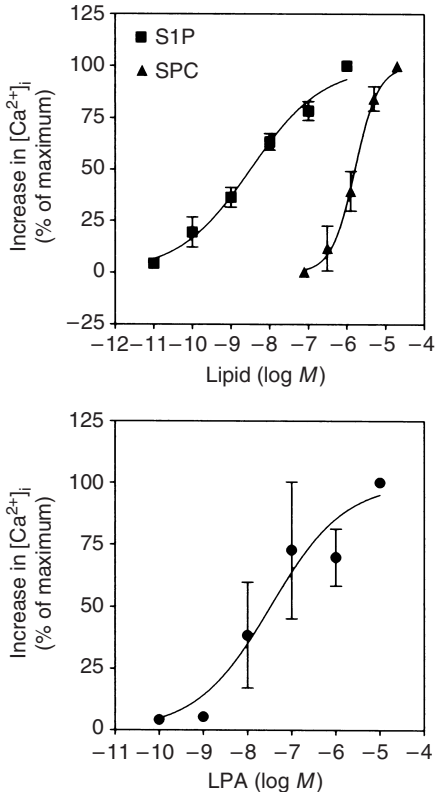

Figure 2. Concentration–response curves of lysophospholipid-induced $[Ca^{2+}]_i$ increases in keratinocytes. The cells were detached, loaded with fura-2, and $[Ca^{2+}]_i$ was measured in suspended cells using a spectrofluorometer. The data points represent mean ± SEM of 4–5 (S1P) or 3 (SPC, LPA) independent experiments.

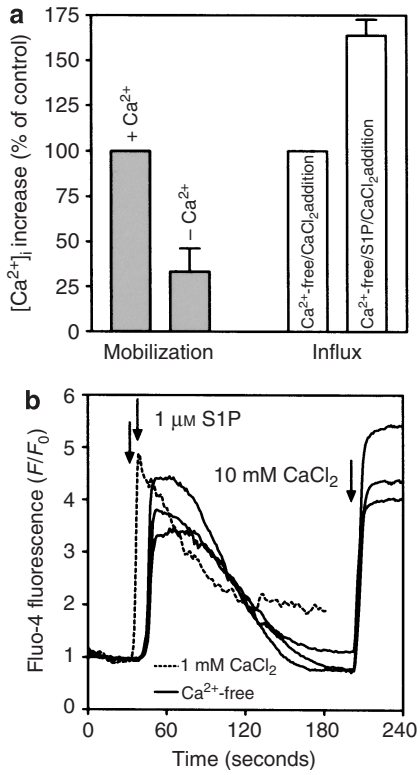

Figure 3. Analysis of S1P-induced $[Ca^{2+}]_i$ increases in the absence and presence of extracellular Ca^{2+}. (**a**) Fura-2 loaded cells in suspension were challenged with 1 µM S1P in the presence of 1 mM $CaCl_2$ ($+Ca^{2+}$) or in Ca^{2+}-free Hank's balanced salt solution containing 50 µM EGTA ($-Ca^{2+}$), and increases in $[Ca^{2+}]_i$ caused by S1P were measured (mobilization). In the experiments performed in Ca^{2+}-free buffer, 1 mM $CaCl_2$ was re-added to cells stimulated previously (Ca^{2+}-free/S1P/$CaCl_2$ addition) or not (Ca^{2+}-free/$CaCl_2$ addition) with 1 µM S1P, and $[Ca^{2+}]_i$ increase caused by Ca^{2+} re-addition was measured (influx). The data represent mean ± SEM of four (mobilization) or three (influx) independent experiments. (**b**) Adherent keratinocytes loaded with fluo-4 were challenged with 1 µM S1P in Ca^{2+}-free medium. Typical courses of $[Ca^{2+}]_i$ after stimulation with 1 µM S1P are shown. The dotted line represents measurements in the presence of 1 mM $CaCl_2$ from the same set of experiments.

reticulum Ca^{2+}-ATPase inhibitor, thapsigargin, on $[Ca^{2+}]_i$ increases by S1P and LPA in keratinocytes. After pretreatment for several minutes with thapsigargin, which by itself caused transient $[Ca^{2+}]_i$ increases (Figure 4a), neither S1P nor LPA were able to elevate $[Ca^{2+}]_i$, indicating that they released Ca^{2+} from endoplasmic reticulum (Figures 4b and c). Furthermore, $[Ca^{2+}]_i$ increases by S1P were inhibited by pretreatment with pertussis toxin. After incubation with 100 ng ml^{-1} pertussis toxin for 16 hours, $[Ca^{2+}]_i$ increases by S1P were 8.9 ± 2.9% of control (mean ± SD, $n = 6$), indicating the involvement of G_i proteins.

To study a possible involvement of phospholipase C (PLC) in Ca^{2+} mobilization by lysophospholipids in adherent keratinocytes, we used the phosphatidylinositol-4,5-bisphosphate/inositol-1,4,5-trisphosphate (IP_3) sensor, cyan fluorescent protein (CFP)-PLCδ1(PH) (Sinnecker and Schaefer, 2004). The use of the PH domain of PLCδ fused to fluorescent proteins has been introduced as early as in 1998/1999, at first to detect changes in phosphatidylinositol-4,5-bisphosphate levels, then for the measurement of IP_3 (Stauffer *et al.*, 1998; Hirose *et al.*, 1999). The suitability of this fluorescent sensor for monitoring IP_3 fluctuations in living cells has been characterized in great detail (Okubo *et al.*, 2001; Xu *et al.*, 2003; Bartlett *et al.*, 2005). This technique has since advanced the understanding of spatiotemporal relationships between the PLC/IP_3 pathway and Ca^{2+} signaling (for review, see Nahorski *et al.*, 2003). As shown in Figure 5, 10 µM LPA (Figure 5a) and 1 µM bradykinin (Figure 5c) caused a decrease of CFP-PLCδ1(PH) fluorescence in the plasma membrane, accompanied by an increase of fluorescence in the cytosol. S1P (1 µM) also induced this process (Figure 5b), indicating that the lysophospholipids, S1P and LPA, stimulated phosphatidylinositol-4,5-bisphosphate hydrolysis and IP_3 generation in keratinocytes. S1P, furthermore, caused a rapid enlargement of the cells, which led to an initial small decrease in cytosolic fluorescence before translocation of CFP-PLCδ1(PH) increased cytosolic fluorescence as in the case of LPA and bradykinin (Figure 5b). These results were confirmed by IP_3 measurements. LPA (10 µM) and S1P (1 µM) increased cellular IP_3 levels by 2.0 ± 0.29-fold and 1.5 ± 0.16-fold within 20 seconds, respectively (mean ± SEM; $n = 3$). Both PLC-dependent and PLC-independent Ca^{2+}

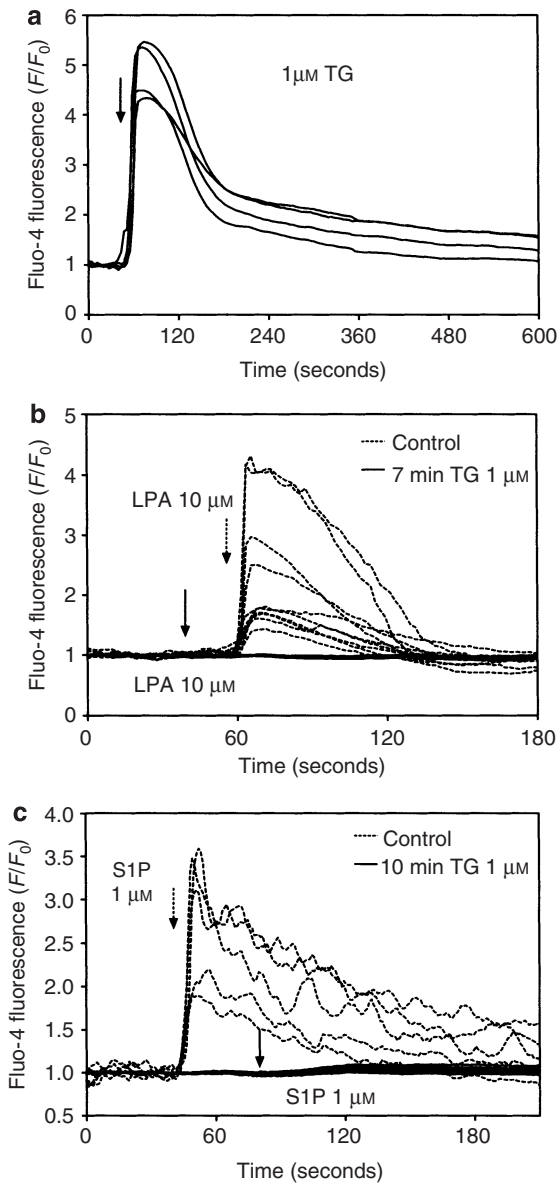

Figure 4. Thapsigargin sensitivity of lysophospholipid-induced $[Ca^{2+}]_i$ increases in keratinocytes. $[Ca^{2+}]_i$ was measured in adherent cells loaded with fluo-4. (**a**) $[Ca^{2+}]_i$ increases induced by 1 μM thapsigargin (TG). (**b**, **c**) $[Ca^{2+}]_i$ increases induced by 1 μM S1P or 10 μM LPA in cells pretreated without (control) or with 1 μM TG for the indicated periods of time.

mobilization has been shown with S1P and LPA. LPA increased $[Ca^{2+}]_i$ in neuroblastoma cells without stimulating IP_3 production (Young *et al.*, 1999, 2000). The same was observed with S1P, for example, in endothelial cells and HEK-293 cells (Meyer zu Heringdorf *et al.*, 1996, 2001). In these cells, Ca^{2+} mobilization is apparently mediated by intracellular S1P, generated by a rapid and transient sphingosine kinase activation (discussed in Meyer zu Heringdorf, 2004). In keratinocytes, however, both lipids stimulated phosphatidylinositol-4,5-bisphosphate hydrolysis and IP_3 generation as in several other cell types.

Keratinocytes express mRNA transcripts of the S1P receptors, $S1P_{1-5}$, and the LPA receptors, LPA_{1-3} (Vogler *et al.*, 2003; Sauer *et al.*, 2004b). For identification of the LPA receptor(s) inducing $[Ca^{2+}]_i$ increases in keratinocytes, we used dioctanoylglycerol pyrophosphate (DGPP 8:0), which is a short-chain derivative of diacylglycerol pyrophosphate. Signaling functions have been assigned to diacylglycerol pyrophosphate in yeast and plants but not in higher animals (reviewed in van Schooten *et al.*, 2006). DGPP 8:0 is a competitive antagonist at human LPA receptors, inhibiting preferentially LPA_3 (K_i ~100 nM) and also LPA_1 (K_i ~7 μM), but not LPA_2 (Fischer *et al.*, 2001; Sardar *et al.*, 2002; Parrill *et al.*, 2004). Pre-incubation with 20 μM DGPP 8:0 shortly before addition of LPA had no influence on $[Ca^{2+}]_i$ increases induced by 100 nM LPA in adherent keratinocytes (Figure 6a). On the other hand, in HEK-293 cells known to express LPA_1 but not LPA_2 (Meyer zu Heringdorf *et al.*, 2001), DGPP 8:0 inhibited $[Ca^{2+}]_i$ increases induced by 100 nM LPA with a pIC_{50} of 5.6 ± 0.07 (mean ± SEM; $n = 5$; Figure 6b). Furthermore, 10 μM DGPP 8:0 shifted the concentration–response curve of LPA-induced $[Ca^{2+}]_i$ increases in HEK-293 cells to the right (Figure 6c), indicating competitive antagonism. From these data, it is concluded that LPA-induced $[Ca^{2+}]_i$ increases in keratinocytes are most likely mediated by the DGPP 8:0-insensitive LPA_2 receptor, whereas those in HEK-293 cells might be primarily mediated by LPA_1.

For identification of the S1P receptor(s) inducing $[Ca^{2+}]_i$ increases in keratinocytes, we used the putative $S1P_3$ antagonist, BML-241 (Koide *et al.*, 2002), the $S1P_1$ agonist, SEW2871 (Sanna *et al.*, 2004), and the $S1P_{1/3}$ antagonist, VPC23019 (Davis *et al.*, 2005). BML-241 was identified by searching a three-dimensional database with pharmacophore models of S1P and subsequently shown to inhibit $[Ca^{2+}]_i$ increases mediated by $S1P_3$ (10 μM BML-241 inhibited ~40% of $[Ca^{2+}]_i$ increases induced by 1 μM S1P), but not $S1P_1$, in a HeLa expression system (Koide *et al.*, 2002). In adherent keratinocytes, 50 μM BML-241 applied 1–2 minutes before S1P fully inhibited $[Ca^{2+}]_i$ increases by 1 μM S1P but did not block the response to 1 μM LPA (Figure 7a). In keratinocytes in suspension, $[Ca^{2+}]_i$ increase by 100 nM S1P in the presence of 50 μM BML-241 was 36 ± 5.7% (mean ± SD; $n = 4$) of those in control cells and thus not fully inhibited (Figure 7b). In HEK-293 cells expressing $S1P_{1-3}$ (Meyer zu Heringdorf *et al.*, 2001), BML-241 inhibited $[Ca^{2+}]_i$ increases by 10 nM S1P with a pIC_{50} of 5.0 ± 0.18 (mean ± SEM; $n = 3$) and a full inhibition at 50 μM (Figure 7d). Analysis of the concentration–response curve of S1P-induced $[Ca^{2+}]_i$ increase revealed a non-competitive mode of inhibition at a BML-241 concentration of 50 μM (Figure 7c). The data obtained with BML-241 must be regarded with caution, as it has been shown recently that BML-241 also inhibits $[Ca^{2+}]_i$ increases by other G-protein-coupled receptors such as P_2 and α_{1A} receptors, and it has been suggested that it might have an influence on the process of Ca^{2+} signaling (Jongsma *et al.*, 2006).

SEW2871 is a $S1P_1$ receptor-selective agonist that is structurally unrelated to S1P (Sanna *et al.*, 2004; Jo *et al.*, 2005). SEW2871 stimulated GTPγS binding to membranes of $S1P_1$-expressing CHO cells (EC_{50} ~10 nM), but not to membranes of cells expressing $S1P_{2-5}$, and mimicked actions

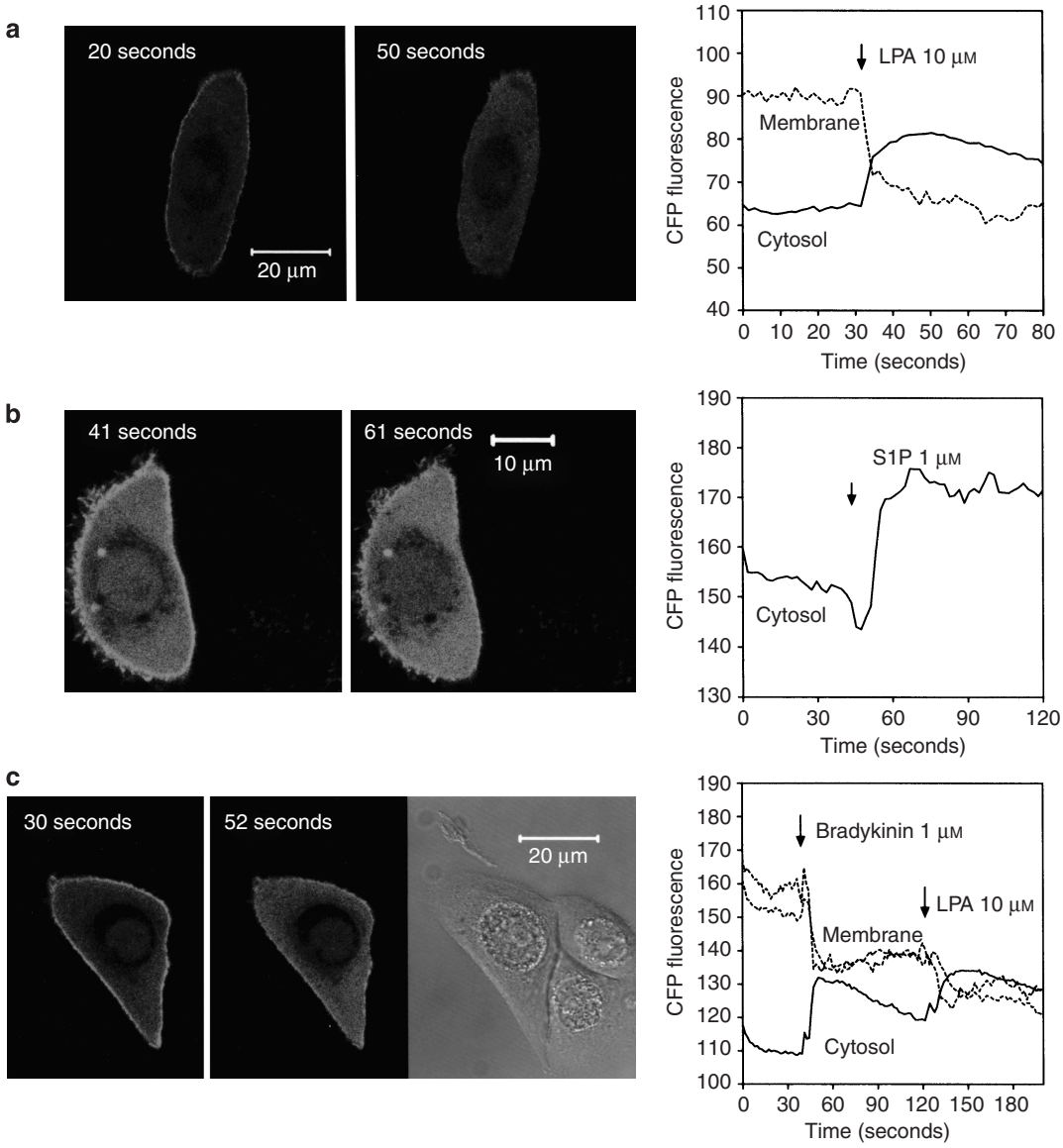

Figure 5. Translocation of the CFP-tagged PH-domain of PLCδ1 (CFP-PLCδ1(PH)) by lysophospholipids in keratinocytes. Cells transfected with CFP-PLCδ1(PH) (Sinnecker and Schaefer, 2004) were grown on glass coverslips and analyzed by confocal microscopy. The images show CFP fluorescence before and after addition of 10 μM LPA (**a**) 1 μM S1P (**b**) or 1 μM bradykinin (**c**). They were taken from time courses that were run at ~1 image per 2 seconds. The traces shown in the right panels represent the mean fluorescence in plasma membranes and cytosol, respectively, obtained from the same measurements as the corresponding images. The time points specified on the images represent the time points at which they were taken. (**b**) After stimulation with 1 μM S1P, cell shape always changed so that it was impossible to quantify CFP fluorescence in the plasma membrane.

of S1P at $S1P_1$, such as phosphorylation of extracellular signal-regulated kinase and acutely transforming retrovirus AKT activation of Rac, stimulation of cell migration, and induction of $S1P_1$ internalization (Sanna *et al.*, 2004; Jo *et al.*, 2005). SEW2871 at 1 μM, a maximal concentration for stimulation of GTPγS binding and extracellular signal-regulated kinase phosphorylation (Sanna *et al.*, 2004; Jo *et al.*, 2005), did not induce $[Ca^{2+}]_i$ increases in adherent keratinocytes (Figures 8a and c) and had no effect on $[Ca^{2+}]_i$ increases by subsequent addition of 1 μM S1P and 10 μM LPA (Figures 8b and d). However, SEW2871 stimulated migration of keratinocytes measured in a modified Boyden chamber with a maximum effective concentration of 1 μM (Figure 8e). SEW2871-stimulated chemotaxis was fully abrogated by $S1P_1$ antisense oligonucleotides (Figure 8e). This observation with a specific $S1P_1$ receptor agonist supports previous studies that have shown that S1P-stimulated migration of keratinocytes was strongly inhibited by pretreatment with $S1P_1$ antisense oligonucleotides (Sauer *et al.*, 2004a). In HEK-293, SEW2981 (1 μM) similarly had no effect on resting $[Ca^{2+}]_i$ but induced internalization of overexpressed $S1P_1$ receptor fused to green fluorescent protein (GFP) (see Supplementary Information). To overcome the concern that $S1P_1$ expression levels might be too low to detect measurable $[Ca^{2+}]_i$ increases by

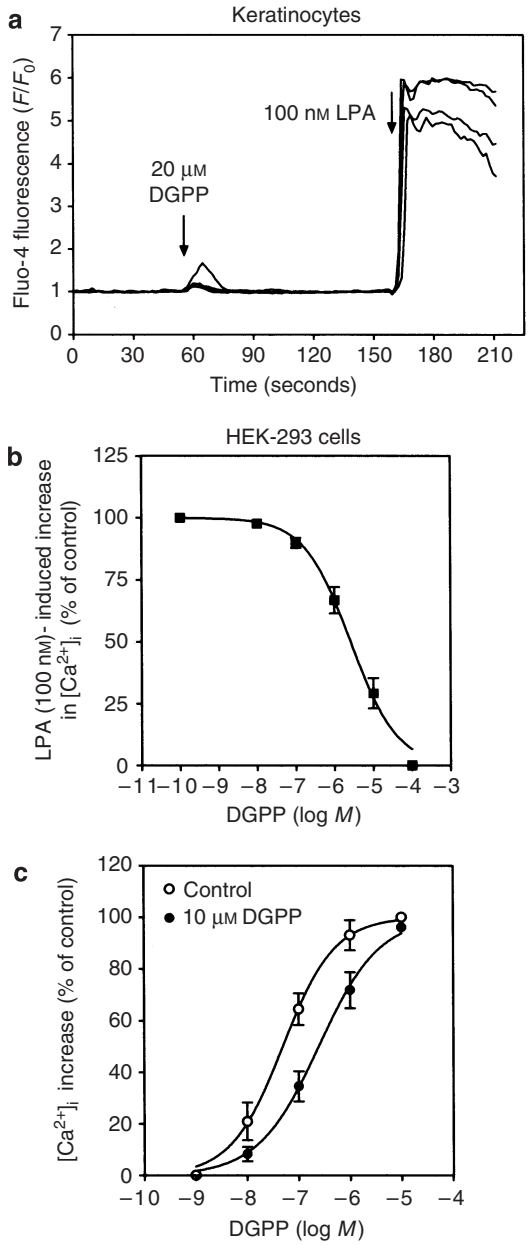

Figure 6. Influence of DGPP 8:0 on $[Ca^{2+}]_i$ increases induced by LPA in keratinocytes and HEK-293 cells. (**a**) $[Ca^{2+}]_i$ was measured in adherent keratinocytes loaded with fluo-4. The cells were treated with 20 μM DGPP 8:0 before addition of 100 nM LPA. (**b**, **c**) $[Ca^{2+}]_i$ was measured in suspended HEK-293 cells loaded with fura-2. The cells were treated for 2 minutes with the indicated concentrations of DGPP 8:0 or solvent before addition of LPA. The data points represent mean ± SEM of 2–4 independent experiments.

SEW2871, we analyzed $[Ca^{2+}]_i$ in HEK-293 cells transiently expressing $S1P_1$-GFP. $S1P_1$-GFP, which is a fully functional $S1P_1$ receptor (Liu *et al.*, 1999), was localized at the plasma membrane in HEK-293 cells where it was found in numerous fine protrusions. SEW2871 did not induce $[Ca^{2+}]_i$ increases in $S1P_1$-GFP-expressing cells. Furthermore, $S1P_1$-GFP-expressing cells, whether treated or not with SEW2871, hardly responded to S1P with any $[Ca^{2+}]_i$ increases (Supplementary Information), in agreement with previous observations suggesting an inhibitory rather than a stimulatory role of $S1P_1$ in Ca^{2+} signaling (Meyer zu Heringdorf *et al.*, 2003b). It is concluded that $S1P_1$ did not contribute to S1P-induced $[Ca^{2+}]_i$ increases in keratinocytes.

VPC23019 was recently synthetized and characterized as a competitive antagonist at $S1P_1$ and $S1P_3$ receptors (Davis *et al.*, 2005). Although it had no stimulatory effect on GTPγS binding in membranes of HEK293T cells expressing $S1P_1$ or $S1P_3$ by itself, VPC23019 caused a rightward shift of S1P-stimulated GTPγS binding in these membranes and displaced bound S1P (Davis *et al.*, 2005). VPC23019 was reported to have no effect on $S1P_2$ (Davis *et al.*, 2005). Addition of 10 μM VPC23019 to adherent keratinocytes induced very small increases in $[Ca^{2+}]_i$ by itself and nearly fully inhibited $[Ca^{2+}]_i$ increases by subsequent addition of 100 nM S1P (Figures 9a and b). In HEK-293 cells, VPC23019 caused a rightward shift of the concentration–response curve of S1P-induced $[Ca^{2+}]_i$ increases (pEC_{50} 8.5 ± 0.17 ($n = 4$) and 7.4 ± 0.24 ($n = 3$), respectively; mean ± SEM; Figure 9c), whereas it had no influence on $[Ca^{2+}]_i$ increases by LPA (Figure 9d). Furthermore, 10 μM VPC23019 inhibited the internalization of $S1P_1$-GFP induced by 100 nM S1P (Figure 9e), which is in agreement with inhibition of $S1P_1$ by VPC23019 (Davis *et al.*, 2005). As $S1P_1$ did not contribute to $[Ca^{2+}]_i$ increases by S1P, it is concluded that $[Ca^{2+}]_i$ increases by S1P in keratinocytes were mediated by $S1P_3$.

Taken together, we have shown that the lysophospholipids, S1P and LPA, not only regulate migration and proliferation of human keratinocytes, but also potently increase $[Ca^{2+}]_i$ in these cells. Using newly available compounds to analyze the lysophospholipid receptor subtypes, we come to the conclusion that the Ca^{2+}-mobilizing actions of S1P and LPA in keratinocytes are mainly mediated by the $S1P_3$ and LPA_2 receptors, respectively. Further studies are needed to clarify the exact role and contribution of the observed $[Ca^{2+}]_i$ increases to keratinocyte functional responses.

MATERIALS AND METHODS

Materials

S1P, SPC, and BML-241 were from Biomol (Biomol GmbH, Hamburg, Germany). LPA, bradykinin, and fatty acid-free BSA were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). SEW2871 was purchased from Calbiochem/Merck Biosciences GmbH (Schwalbach, Germany), whereas VPC23019 and DGPP 8:0 were from Avanti Polar Lipids Inc. (Alabaster, AL). Fura-2 and Fluo-4 were obtained from Molecular Probes/Invitrogen GmbH (Karlsruhe, Germany). All other materials were from previously described sources (Ruwisch *et al.*, 2001; Meyer zu Heringdorf *et al.*, 2003a, b; Vogler *et al.*, 2003; Sauer *et al.*, 2004a). Stock solutions of S1P and SPC were made in methanol. Aliquots thereof were dried down and dissolved in 1 mg ml^{-1} fatty acid-free BSA. SEW2871 and BML-241 were dissolved in dimethyl sulfoxide, DGPP 8:0 in methanol, and LPA in H_2O, and all were further diluted in H_2O. VPC23019 was dissolved in dimethyl sulfoxide/1 N HCl (95/5, v/v) and further diluted in 1 mg ml^{-1} fatty acid-free BSA. The respective solvents were used as control.

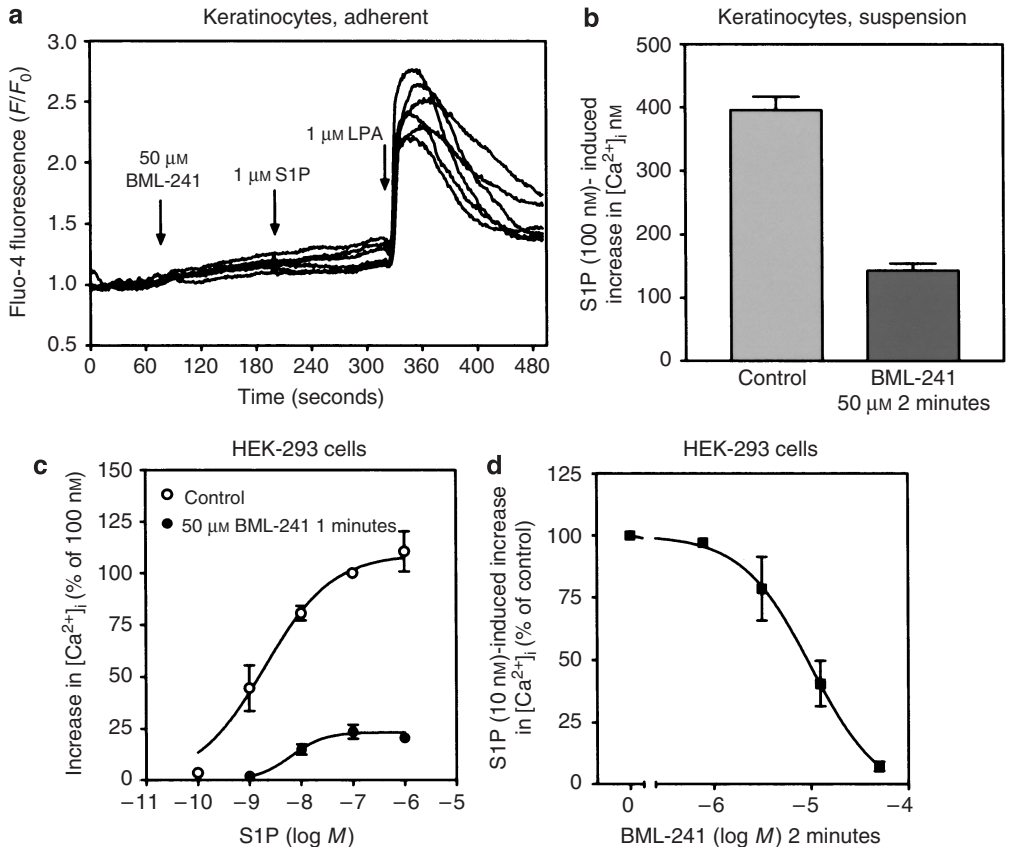

Figure 7. Influence of BML-241 on $[Ca^{2+}]_i$ increases induced by S1P in keratinocytes and HEK-293 cells. (**a**) $[Ca^{2+}]_i$ was measured in adherent keratinocytes loaded with fluo-4. The cells were treated with BML-241, S1P, and LPA as indicated. (**b**–**d**): $[Ca^{2+}]_i$ was measured in suspensions of (**b**) keratinocytes or (**c**, **d**) HEK-293 cells loaded with fura-2. The cells were treated with the indicated concentrations of BML-241 or solvent before addition of S1P. The values represent mean ± SD, (**b**) $n = 4$ or (**c**, **d**) mean ± SEM of 3–5 experiments.

Cell culture and transfection

Keratinocytes from human foreskin were isolated and cultivated as described previously (Vogler *et al.*, 2003). Briefly, the cells were grown in serum-free keratinocyte growth medium (Cambrex Corporation, now Lonza, Verviers, Belgium) containing bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, gentamicin, and amphotericin B (Vogler *et al.*, 2003) in a humidified atmosphere of 5% CO_2/95% air at 37°C. Transfection of keratinocytes was performed with Fugene 6 Transfection Reagent (Roche Diagnostics GmbH, Applied Science, Mannheim, Germany) according to the manufacturer's instructions. For downregulation of the $S1P_1$ receptor, keratinocytes were transfected with oligonucleotides as indicated for 3 days at a final concentration of 0.1 μM ($S1P_1$ antisense oligonucleotides, 5′-AGTGGACACCATAGCTGCTAA-3′; $S1P_1$ control oligonucleotides, 5′-TTAGCACGTATGGTGTCCACT-3′). Relative abundance of the $S1P_1$ receptor protein expressed after antisense oligonucleotide pretreatment was assessed by western blot analysis. HEK-293 cells were cultivated and transfected as described previously (Meyer zu Heringdorf *et al.*, 2003b) and kept for 24 hours in serum-free medium before experiments.

Quantitative real-time PCR

Keratinocytes were cultured in keratinocyte growth medium and then stimulated with S1P (1 μM) or LPA (1 μM) for 5 days. RNA was collected using RNeasy kit (Qiagen GmbH, Hilden, Germany) and cDNA was generated from total RNA using the FermentasAid First strand cDNA synthesis kit (Fermentas GmbH, St Leon-Rot, Germany) according to the manufacturer's instructions. Quantitative real-time PCR was performed using a LightCycler480 and the SYBR Green PCR master mix (Roche Diagnostics GmbH). Cyclophilin A was used as a normalization control for all experiments. For the measurement of the differentiation markers keratin 1, keratin 10, and involucrin, the following primers were used: keratin 1, CTATGGACAA CAACCGCA (forward), CTTTGAATTTCTCACACTAT CCC (reverse); keratin 10, AAGGAACTGACTACAGAAATTGATAATA (forward), GCTGCACACAGTAGCGA (reverse); involucrin, GGAATTATTGTC CAGTGCCA (forward), GGATTTATTATGTATCATTGTACACACTC (reverse); cyclophilin A, TTTGCTTAATTCTACACAGTACTTAGAT (forward), CTACCCTCAGGTGGTCTT (reverse). Total RNA of three different sets of keratinocytes was used to analyze gene expression. Data were obtained in triplicate and specific mRNA levels were expressed as mean + SEM relative to non-stimulated cells.

Measurement of intracellular Ca^{2+} concentration

In adherent cells, $[Ca^{2+}]_i$ was measured by fluo-4 fluorescence imaging as described previously (Meyer zu Heringdorf *et al.*, 2003a). Human keratinocytes or HEK-293 cells grown on glass coverslips were loaded with 2 μM fluo-4/AM for 30 minutes. Measurements were performed in Hank's balanced salt solution (118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 15 mM

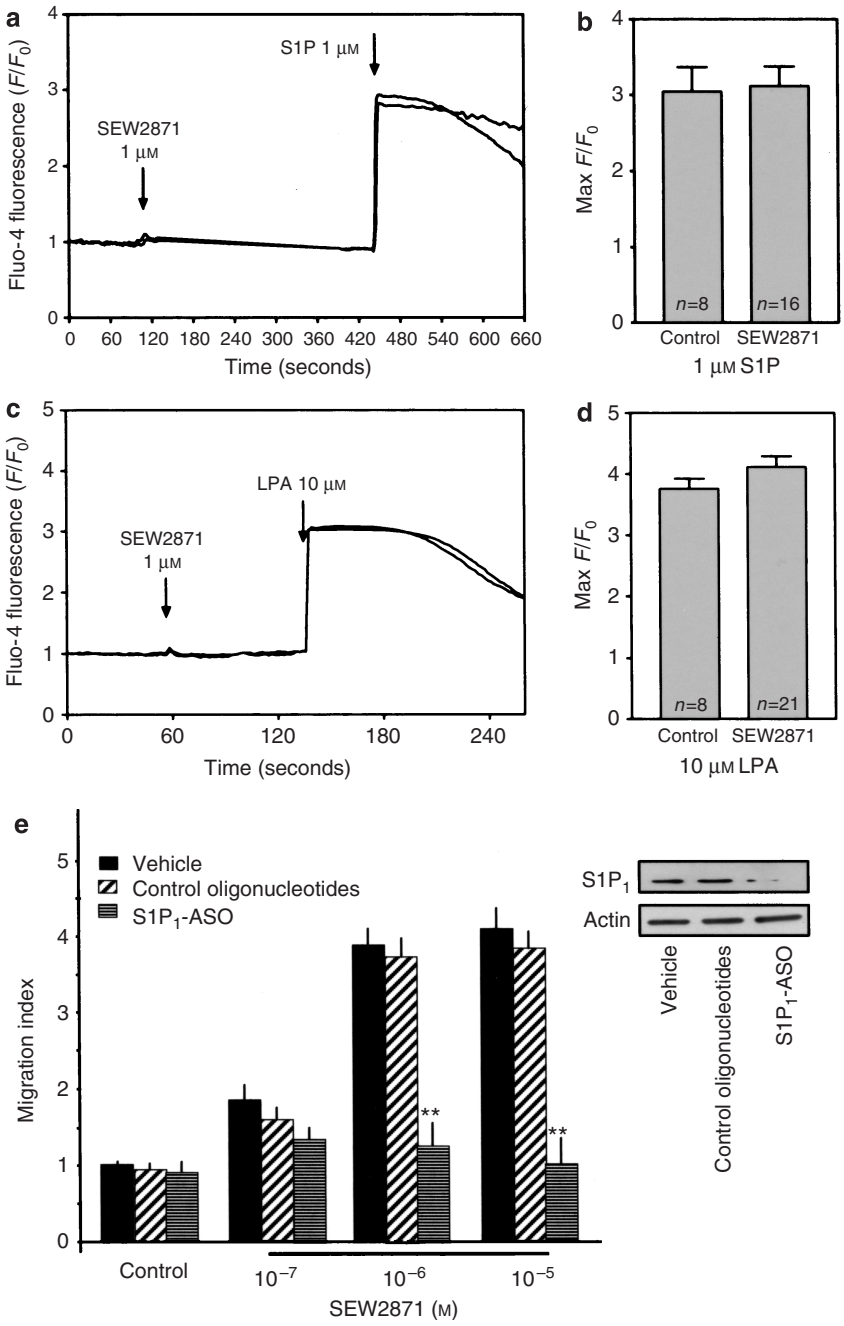

Figure 8. Influence of SEW2871 on $[Ca^{2+}]_i$ and chemotactic activity of keratinocytes. (**a**, **c**) $[Ca^{2+}]_i$ was measured in adherent keratinocytes loaded with fluo-4. The cells were treated with SEW2871, S1P, and LPA as indicated. (**b**, **d**) Quantification of S1P- and LPA-induced $[Ca^{2+}]_i$ increases in adherent keratinocytes treated with 1 μM SEW2871 or solvent (control) for 2–5 minutes before addition of S1P or LPA, as shown in (**a**, **c**). The data represent maximum values of normalized fluo-4 fluorescence (max F/F_0), shown as mean ± SEM of the indicated number of cells. (**e**) Migration of human keratinocytes toward SEW2871 in the presence of $S1P_1$ antisense or control oligonucleotides. The cells were pre-incubated with $S1P_1$-specific antisense oligonucleotides ($S1P_1$-ASO) or control oligonucleotides for 3 days at a final concentration of 0.1 μM. Then, migration assays were performed. Values are expressed as mean ± SD ($n = 3$). Downregulation of the $S1P_1$ receptor was verified by western blotting.

HEPES, pH 7.4) at room temperature. Fluo-4 fluorescence was imaged with an inverted confocal laser scanning microscope (Zeiss LSM510 or Leica TCS SP2). For excitation, the 488 nm line of an argon laser was used, whereas emission was recorded at wavelengths above 505 nm. Mean fluorescence of single cells was calculated by averaging regions of interest. Measurements of $[Ca^{2+}]_i$ with the ratioable dye, fura-2, were performed in a Hitachi F2000 spectrofluorometer using cells in suspension (Meyer zu Heringdorf *et al.*, 2003b). Briefly, monolayers of keratinocytes were detached with trypsin and loaded with 1 μM fura-2/AM in Hank's balanced salt solution for 1 hour at room temperature. Thereafter, the cells were washed with Hank's balanced salt solution, resuspended at a density

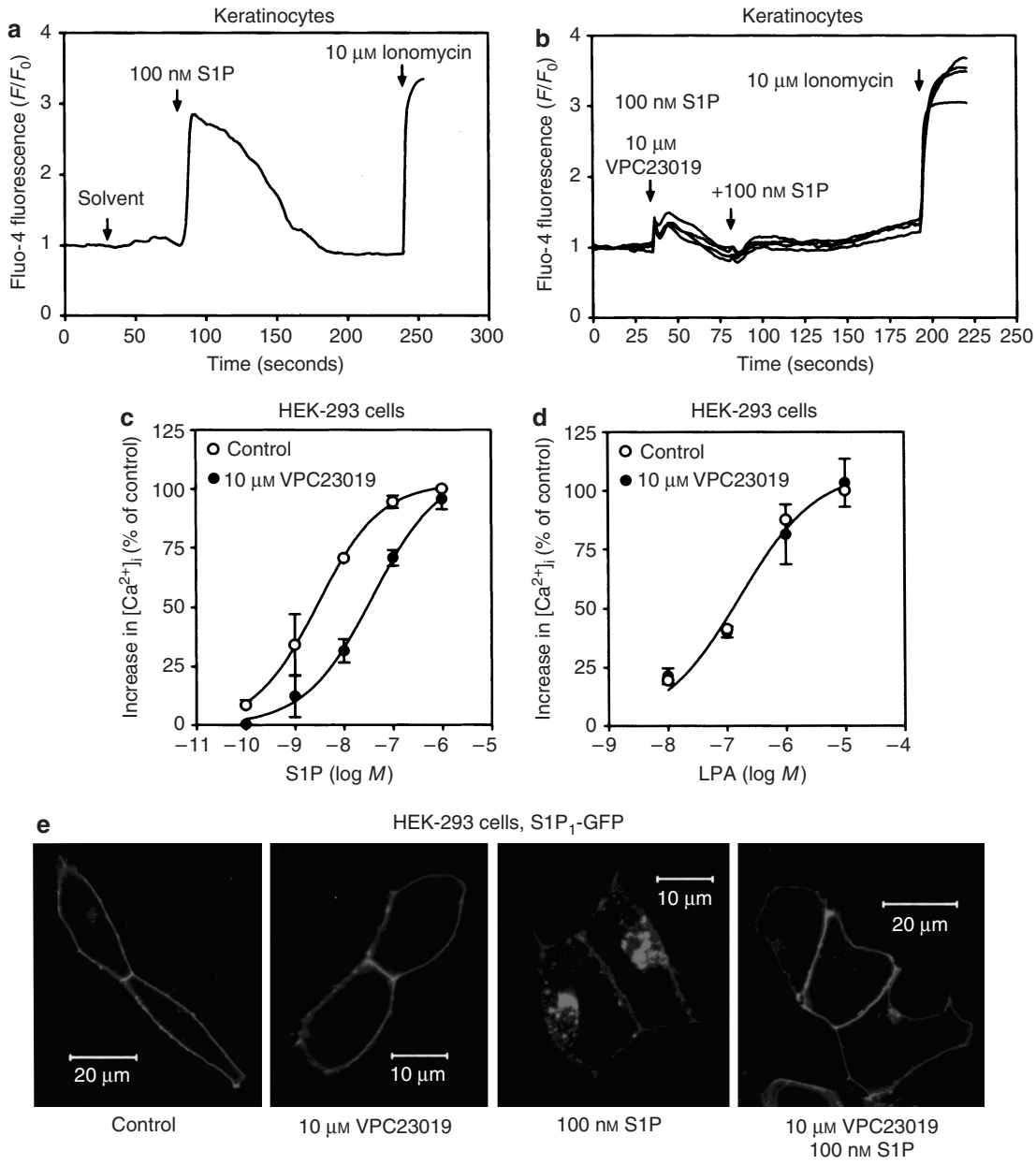

Figure 9. Influence of VPC23019 on $[Ca^{2+}]_i$ and localization of $S1P_1$. (**a**, **b**) $[Ca^{2+}]_i$ was determined in adherent, fluo-4-loaded keratinocytes that were challenged as indicated with VPC23019, S1P, and ionomycin. (**c**, **d**) Analysis of concentration–response curves of S1P- and LPA-induced $[Ca^{2+}]_i$ increases in fura-2-loaded HEK-293 cells. VPC23019 or its solvent were added 2 minutes before stimulation. The values represent mean ± SEM of 3–4 independent experiments. (**e**) Inhibition of S1P-induced $S1P_1$-GFP internalization by VPC23019. $S1P_1$-GFP-expressing HEK-293 cells were treated for 2 hours as indicated.

of 1×10^6 cells ml^{-1}, and used for fluorescence measurements at room temperature within the next hour.

Imaging of CFP and GFP fluorescence

The fluorescence of cells transfected with CFP-PLCδ1(PH) or $S1P_1$-GFP was analyzed by confocal laser scanning microscopy with a Zeiss LSM510 microscope and a ×63/1.4 oil immersion objective. CFP fluorescence was monitored with excitation at 458 nm and emission measured with a 475-nm-long pass filter. GFP was analyzed with excitation at 488 nm and emission measured with a 505–550 band pass filter.

Measurement of IP_3 formation

Keratinocytes grown in 3.5 cm dishes were stimulated for 20 seconds with 10 μM LPA, 1 μM S1P, or the respective vehicles. The reaction was stopped with 0.5 ml of 0.5 M trichloroacetic acid, and supernatants were extracted three times with 3 ml of water-saturated diethyl ether. The neutralized samples (200 μl) were complemented with 50 μl of EDTA (30 mM) and 50 μl of $NaHCO_3$ (60 mM). IP_3 mass was measured by competition with $[^3H]IP_3$ (PerkinElmer Life and Analytical Sciences, Rodgau—Jügesheim, Germany; 792 GBq $mmol^{-1}$) using IP_3 binding protein prepared from bovine adrenal cortex as described previously (Chilvers *et al.*, 1991).

Measurement of keratinocyte migration

Migration of keratinocytes in response to a gradient of SEW2871 was measured in a modified Boyden chamber. The cells were added to the upper wells of the chamber. The lower wells, separated by a fibronectin-coated membrane, contained SEW2871 at the indicated concentrations. The keratinocytes that had migrated through the membrane were fixed, stained by GIEMSA, and quantified by light microscopy at a magnification of $\times 150$ by counting the stained cells from four randomly selected fields. All samples were tested in triplicates and the data are expressed as the mean ± SD. The specific migration index following stimulation was calculated as the ratio after setting the control vehicle random migration at 1.0.

Data presentation and analysis

Traces of $[Ca^{2+}]_i$ and fluorescence images shown in the figures are representative for at least three similar experiments. Averaged data are mean ± SEM from the indicated number (*n*) of experiments or mean ± SD from a representative experiment with the indicated number (*n*) of replicates. Non-linear regression analysis of concentration–response curves and statistical tests were performed using the Prism program (GraphPad Software).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. Influence of SEW2871 on $[Ca^{2+}]_i$ and localization of $S1P_1$ in HEK-293 cells.

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