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Characterization of the transient receptor potential channels mediating lysophosphatidic acid-stimulated calcium mobilization in B lymphoblasts

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Abstract

Altered 1-oleoyl-lysophosphatidic acid (LPA, 100 μM)-stimulated calcium responses occur in B-lymphoblast cell lines from bipolar disorder patients, but the mechanism(s) involved is uncertain. Lysophosphatidic acid shares a structurally similar fatty acid side chain with the diacylglycerol analogue, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), a known activator of subtypes 3, 6 and 7 of the canonical transient receptor potential (TRPC) cation channel subfamily. Accordingly, the objective of this study was to determine whether the LPA-stimulated calcium response in B-lymphoblasts is mediated, in part, through this TRPC channel subfamily. Divalent cation selectivity in response to thapsigargin, LPA and OAG were used to distinguish TRPC-like character of the responses to these agents in BLCLs. The sensitivity to gadolinium, an inhibitor of capacitative calcium channels, was used to determine the store-operated nature of the responses. The TRPC isoforms that are present in BLCLs as identified by immunoblotting and/or PCR include TRPC1, 3 and 5. Minimal barium influx in calcium-free buffer was observed following thapsigargin stimulation. However, LPA stimulated barium influx of a magnitude similar to that induced by OAG. Thapsigargin-provoked calcium influx was completely inhibited by gadolinium (10 μM), whereas LPA and OAG-stimulated responses were partially inhibited and potentiated, respectively. The results suggest that 100 μM LPA stimulates calcium entry through channels with characteristics similar to TRPC3, as TRPC6 and 7 are absent in B-lymphoblasts.
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Keywords: Signal transduction; Ca^{2+} signalling; Transient Receptor Potential (TRP) channels; B lymphoblasts; Lysophosphatidic acid; 1-oleoyl-2-acetyl-*sn*-glycerol (OAG); Bipolar disorder

Introduction

Surrogate cell models, such as B-lymphoblast cell lines (BLCLs) (Emamghoreishi et al., 1997), leukocytes, platelets (Warsh et al., 2004) and superior olfactory nasal epithelium (Hahn et al., 2005) derived from patients with bipolar disorder (BD) have provided critical cellular matrices to elaborate the nature of the underlying molecular mechanisms that may account for disrupted Ca^{2+} homeostasis in BD (for review see Warsh et al., 2004). Findings of higher basal and agonist-stimulated intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), first reported in platelet and leukocyte preparations from BD patients compared with healthy subjects (Dubovsky et al., 1989), were extended to BLCLs (Emamghoreishi et al., 1997; Warsh et al., 2004), which, by virtue of repeated passage ex vivo in culture, are freed from the effects of medication and neurohormonal stress that might impact on intracellular Ca^{2+} homeostasis in vivo. That altered intracellular Ca^{2+} homeostasis reflects an important aspect of the pathophysiology of BD received further support in the observations that therapeutically relevant concentrations of the prototypical mood stabilizer, lithium, attenuated agonist (lysophosphatidic acid [LPA]) stimulated Ca^{2+} responses in BLCLs treated chronically in vitro

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(Wasserman et al., 2004). Collectively, these observations imply that a disturbance in receptor-activated signalling and induction of intracellular Ca^{2+} mobilization may occur in BD (Warsh et al., 2004). However, the specific nature and site of the disturbance(s) within this receptor-activated Ca^{2+} signalling cascade are uncertain.

The LPA-stimulated Ca^{2+} mobilization is thought to be mediated in a variety of cell types, in part, through G-protein coupled receptor (GPCR) activation of phosphatidylinositol (PI) hydrolysis, referred to as receptor-operated Ca^{2+} (ROC) entry (Anliker and Chun, 2004), but this has not been clearly established for the BLCLs used as a disease reporter model for BD. In this respect, the maximal stimulating concentration of LPA-induced Ca^{2+} mobilization in BLCLs was determined to be ∼100 μM (Wasserman et al., 2004) (but see Rosskopf et al., 1998), whereas maximal GPCR-mediated activation by LPA has been reported in other cell types at low μM concentrations (∼10 nM–10 μM) (Ohta et al., 2003; Rosskopf et al., 1998; Segura et al., 2004). This range is consonant with the maximal stimulating concentration of LPA (∼24 nM) determined using A431 epidermoid carcinoma cells as a methodological reference preparation with the same conditions as for BLCLs (Wasserman, 2003). In light of these observations, the mechanism by which LPA-stimulates Ca^{2+} responses in BLCLs is likely more complex than initially conceived, possibly involving multiple channel types and Ca^{2+} entry mechanisms. In addition, LPA-mediated Ca^{2+} influx has been described as insensitive to both phospholipase C (PLC) and $G_{i/o}$ inhibitors, U-73122 and pertussis toxin (PTX), respectively, in both human monocytic cells (U937) (Lee et al., 2004) and neutrophils (Itagaki et al., 2005). Therefore, the mode of LPA action in these cell lines seems to be independent of GPCR activation, which also raises questions about the actual mechanisms underlying the Ca^{2+} responses initiated by LPA in these cells. Of note, LPA shares a structurally similar fatty acid side chain with the cell permeant diacylglycerol analogue, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), which is a known activator of the canonical transient receptor potential (TRPC) 3/6/7 subfamily of channels (Hofmann et al., 1999). Therefore, LPA may activate intracellular Ca^{2+} fluxes by at least two distinct pathways, one that is GPCR-dependent and the other by a ligand-gated mechanism stimulating TRPC3/6/7 channels directly, similar to OAG. The TRPC channels, expressed in non-excitable as well as neural cell types (Hofmann et al., 2002; McKay et al., 2000; Riccio et al., 2002; Sossey-Alaoui et al., 1999), are thought to function as store-operated and/or receptor-operated channels to allow non-selective cation passage (Clapham et al., 2001; Vazquez et al., 2004; Vennekens et al., 2002).

Accordingly, LPA-mediated Ca^{2+} signalling was further characterized in this cell model to elucidate the specific pathways of Ca^{2+} mobilization involved. Indeed, comparing the divalent cation selectivity and gadolinium (a capacitative Ca^{2+} channel inhibitor) sensitivity of thapsigargin-, LPA- and OAG-stimulated responses, together with immunoblotting and reverse transcriptase-polymerase chain reaction (RT-PCR), we show that LPA at 100 μM stimulates Ca^{2+} entry, in part, through TRPC3-like channel activity in BLCLs.

Materials and methods

Cell culture

Lymphocytes were isolated from 10–20 mL of anticoagulated (acid citrate dextrose) blood, obtained from physically healthy subjects as previously described (Emamghoreishi et al., 1997). After centrifugation over Histopaque (Sigma–Aldrich, Oakville, ON), an enriched leukocyte fraction was treated with Epstein Barr virus to transform and immortalize the contained B lymphocytes using standard techniques (Emamghoreishi et al., 1997). The BLCLs were grown in RPMI-1640 medium, supplemented with 15% foetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1 mM sodium pyruvate (Wasserman et al., 2004). Undifferentiated PC12 cells (ATCC, Manassas, VA) were grown in RPMI-1640 medium supplemented with 5% FBS, 10% horse serum, 1% L-glutamine, 1% penicillin/streptomycin in 75 cm^2 flat bottom polystyrene flasks. All cell culture materials, except those noted, were from Gibco (Burlington, ON). All chemicals were of analytical grade.

Immunoblotting

Detection of the TRPC isoforms present in BLCLs was carried out using standard immunoblotting protocols and commercially available antibodies, as previously described (Andreopoulos et al., 2004). TRPC1 was detected as previously described (Andreopoulos et al., 2004). Homogenates of human frontal cortex, (Brodmann area 9), which was known to express TRPC1, TRPC3, TRPC4, and TRPC5, were used as a positive control (Hofmann et al., 2000; Thebault et al., 2005). In addition, HEK293 cells transiently overexpressing TRPC3 were also used as a positive control. Pooled BLCLs from several subjects (1×10^8 cells) were sonicated in lysis buffer containing protease inhibitors, and protein concentration was determined. Lysates were boiled in denaturing sample buffer and loaded onto SDS-PAGE gels, then transferred to PVDF membranes (Perkin–Elmer, Woodbridge, ON) using standard methods (Andreopoulos et al., 2004). Membranes were blocked with 0.5% egg white albumin (for TRPC3 detection) or 5% bovine serum albumin (for TRPC4 and 5 detection) in phosphate buffered saline. Anti-TRPC3 primary antibody (ACC-016, Alamone Laboratories, Israel) was used at a concentration of 1:500 and an incubation time of 2 h. Next, HRP-conjugated protein A (Bio-Rad Laboratories Inc., Hercules, CA) was used as the secondary antibody at a dilution of 1:3000 for 40 min. Anti-TRPC4 and anti-TRPC5 primary antibodies (ACC-018 and ACC-020, respectively, Alamone Laboratories, Israel) were used at a concentration of 1:200 and an incubation time of 2.5 h. The secondary antibody, HRP-conjugated rabbit IgG (Vector Labs, Burlington, ON), was used at a dilution of 1:3000 with 40 min incubation, and visualization was carried out using ECL (Amersham Pharmacia, Baie d'Urfe, QC) and chemiluminescence. Control peptide antigens, used to compete out respective putative TRPC immunoreactive bands as part of the test of antibody specificity, were obtained from the supplier.

RT-PCR detection of TRPC6 and TRPC7 transcripts

The presence of TRPC6 and TRPC7 transcripts in pooled BLCLs and frontal cortex was determined using RT-PCR. Total RNA was extracted from 1×10^7 frozen cells using an RNeasy® kit (QIAGEN, Mississauga, ON) and subsequently treated with DNase I. The RNA quality, purity and integrity were confirmed and concentrations determined as previously described (Andreopoulos et al., 2004). First-strand cDNA synthesis was also carried out as previously reported (Andreopoulos et al., 2004). To create a negative control, one sample was processed in the absence of reverse transcriptase. Gene-specific primer pairs were designed using Primer Express® v2.0 (Applied Biosystems, Foster City, CA) to amplify all TRPC7 splice variants (GenBank accession nos. Full-length cDNA- AJ421783, Splice Variant A-AJ549088, Splice Variant B-AJ549089, Splice Variant C-AJ549090); sense 5′-AAGGCCAAAAGCTGTGA AAA-3′ and antisense 5′-CACCCTCAGGTGGTCTTTGT-3′, with an expected product size of 363 bp. The TRPC6 transcript was amplified using the primer pairs as described by Andreopoulos et al. (2004), and the expected size of the amplicon was 98 bp. Each 50 μL PCR reaction mixture contained 1X PCR buffer, 200 μM dNTPs, 0.8 μM of each primer pairs, 1.25 U Taq DNA polymerase (MBI Fermentas, Burlington, ON), and 1.5 mM $MgCl_2$. The PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s with a final extension phase at 72 °C for 10 min (MJ Research PTC-260). The amplification products were separated on a 1% agarose gel containing ethidium bromide with a 100 bp standard ladder (New England Biolabs Ltd., Pickering, ON) for size comparison. All PCR materials, except those noted, were from Invitrogen (Burlington, ON).

Agonist-stimulated Ca^{2+}/Ba^{2+} mobilization

Cells ($1–3\times10^6$/mL) were incubated with 1 μM fura-2 acetoxymethylester (fura 2-AM, Molecular Probes, Eugene, OR) or an equivalent volume of DMSO for autofluorescence determination, in RPMI-1640 (30 min, 37 °C). After washing with RPMI 1640 and with HEPES buffered saline (HEPES: 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH = 7.4), cells were then resuspended at 1×10^6 cells/mL, transferred to a cuvette, and equilibrated (3 min, 37 °C) in the temperature-controlled chamber of the fluorometer (Perkin–Elmer LS50B). In experiments conducted with cation as a variable, Ca^{2+}-free buffer was used and the appropriate cation, either Ca^{2+}, Ba^{2+}, or vehicle (i.e. H_2O), was added to the cuvette prior to stimulation. After measuring a stable basal fluorescence ratio (R, excitation 340/380 nM, emission 500 nM) for 60 s, the agent of interest (LPA [Sigma–Aldrich, Oakville, ON], OAG [Calbiochem, La Jolla, CA] or 2,4,6-Trimethyl-*N*-(*m*-3-trifluoromethylphenyl)benzene-sulfonamide [*m*-3M3FBS; Calbiochem]) was added. Fluorescence ratios were then measured for 4 min (5.5–7 min for assays with OAG) to capture peak responses, and decay or plateau phases. Results are reported as an autofluorescence corrected ratio of 340/380 for both Ca^{2+} and Ba^{2+} experiments.

Thapsigargin (TG) activation of store-operated Ca^{2+} (SOC) channels

Cells ($1–3\times10^6$/mL) were loaded with fura-2 AM in RPMI-1640 (45 min, 37 °C), as above. Cells were washed once with RPMI and three times with Ca^{2+}-free HEPES, resuspended at 1×10^6 cells/mL and equilibrated (37 °C, 3 min). After determining basal R, cells were stimulated with TG (200 nM, Alamone, Israel) and R was measured for 4 min to allow for transient ER pool depletion (Wasserman et al., 2004). The Ca^{2+}/Ba^{2+} concentration in the buffer was then raised to 1 mM with $CaCl_2$ /$BaCl_2$ and R measured for up to 5 min to monitor the Ca^{2+}/Ba^{2+} entry.

Effect of the phospholipase C (PLC) inhibitor U-73122 on LPA-induced $[Ca^{2+}]_i$ responses

The inhibitor, U-73122 (Calbiochem) and its inactive analogue, U-73343 (Calbiochem), were dissolved in DMSO to a concentration of 0.5 mg/mL and mixed at 40 °C for 40 min immediately prior to experiments. Aliquots of cells were loaded with fura-2 AM as above and preincubated with U-73122 (4–8 μM) or U-73343 (4–8 μM) for 10 min preceding stimulation with LPA.

Effect of Pertussis Toxin (PTX) on LPA-induced $[Ca^{2+}]_i$ responses

Cells (1×10^6/mL) were maintained in RPMI containing PTX (200 ng/mL, List Biologicals, Campbell, CA), or vehicle for 24 h and then washed twice in RPMI, prior to the initiation of dye loading and the LPA-stimulated Ca^{2+} mobilization, as described above. Percent inhibition of Ca^{2+} mobilization was determined by comparing the increase in LPA-stimulated intracellular Ca^{2+} levels in equivalent aliquots of cells treated with or without PTX.

Data analysis

Statistical analysis was carried out using the SPSS© statisical software package (version 11.0). Differences between stimulation conditions in dependent measures including baseline, peak and delta (Δ = peak-baseline) fluorescence ratios (R) were tested using unpaired Student's *t*-test for comparison of two groups or one-way ANOVA for comparison of multiple conditions, with cation (Ba^{2+} versus Ca^{2+}) or drug as independent variables. Post-hoc comparison of group means was carried out using the Tukey's HSD test. Due to the nature of the response to LPA stimulation in the presence of Ba^{2+} (Fig. 3B), area under the curve (AUC) measurements (area measured from point of drug addition [60 s] to end of measurement [350 s]) were used as the dependent variable. Statistics with p values <0.05 were taken as significant.

Results

Occurrence of TRPC subtypes in BLCLs

We have previously reported the presence of TRPC1 and 3 (Fig. 1A) protein in BLCLs using standard immunoblotting

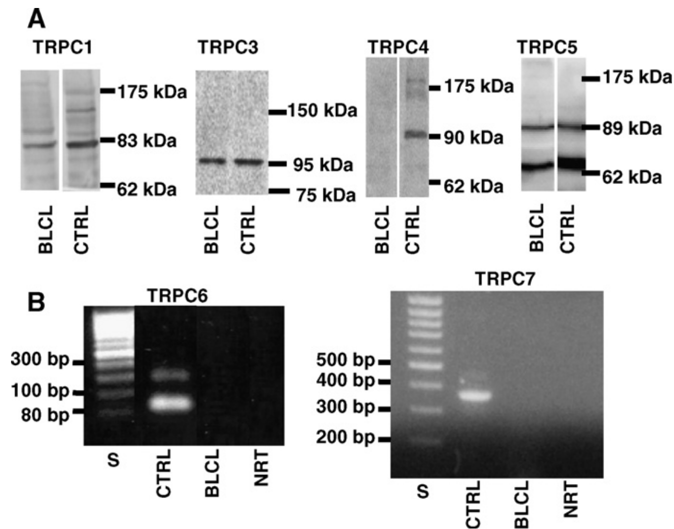

Fig. 1. TRPC1, 3 and 5, but not TRPC4, 6 or 7 are expressed in BLCLs. A. TRPC1, 3 and 5 immunoreactive bands were detected in pooled BLCLs from multiple subjects and reference control tissues migrating at single bands of the respective masses; 83, 95, and 89 kDa. TRPC4 was detected in human frontal cortex homogenate as a single immunoreactive band migrating at the expected MW of ~ 90 kDa, but was not detected in BLCL lysates. B. Primers designed to detect a 363 bp amplicon of TRPC7 identified a single product of the expected size in human frontal cortex but not in BLCLs. S indicates the 100 bp DNA ladder, CTRL is the positive control (frontal cortex) and NRT is a negative control (no reverse transcription).

techniques (Andreopoulos et al., 2004). For completeness, Fig. 1 includes an independent replication of the above findings combined with the other TRPC subtypes examined in this study, to provide a comprehensive panel demonstrating those that are expressed in BLCLs. Using antisera directed against specific peptide sequences unique to individual TRPC subtypes and human frontal cortex as a positive control, prominent immunoreactive bands were detected at approximately 80 kDa for TRPC1, 95 kDa for TRPC3, and 89 kDa for TRPC5 (Fig. 1A). As an additional positive control for TRPC3, samples from HEK293 cells overexpressing TRPC3 produced an immunoreactive band migrating with a similar mass as that detected in lysates from both human frontal cortex and BLCLs (data not shown). In contrast, no immunoreactive bands could be detected corresponding to TRPC4 in BLCLs, unlike the human frontal cortex, in which an immunoreactive band migrating at the expected mass of approximately 90 kDa for TRPC4 was observed (Flockerzi et al., 2005). The putative TRPC4 and TRPC5 immunoreactive bands were successfully competed out by the addition of the antigen against which the respective antibodies were raised (data not shown). Using RT-PCR, no mRNA for TRPC6 or TRPC7 was detected in BLCLs, although amplicons of expected size (90 and 363 bp, respectively) were observed in the human frontal cortex (Fig. 1B). Therefore, no immunoblotting was performed for TRPC6/7 in BLCLs.

OAG and LPA stimulated Ca^{2+} response in BLCLs

Having determined that TRPC3 is essentially the only member of the TRPC3/6/7 subfamily expressed in BLCLs, we then addressed the question of whether LPA activates Ca^{2+} influx through TRPC3 and in an OAG-like manner in BLCLs. Thus, it was shown that OAG stimulated Ca^{2+} mobilization in BLCLs in a concentration-dependent manner over the range of 50–300 μM (Fig. 2A). Reasoning that if LPA acted through an OAG-like mechanism to mediate Ca^{2+} influx, there should be no additivity of the responses, we measured the stimulatory effects of LPA and OAG alone and in combination. As shown in Fig. 2B, simultaneous addition of LPA (100 μM) and OAG (100 μM) resulted in a Ca^{2+} response that was larger in magnitude than OAG alone ($F=149$, $df=2,10$, $p<0.001$; Tukey $p<0.001$) but lower than that obtained following stimulation with LPA alone (Tukey $p=0.001$).

Characterization of divalent cation selectivity in LPA, OAG and m-3M3FBS responses

In the absence of specific agonists or antagonists for the TRPC family, the selectivity of these channels to divalent cations is one of the methods available to discern their involvement in intracellular Ca^{2+} responses. The Ca^{2+} release-activated Ca^{2+} (CRAC) channels are highly Ca^{2+} selective members of the SOC channel family (Hoth and Penner, 1992). In contrast, TRPC channels are non-selective cation channels that allow Ba^{2+} permeation (Vazquez et al., 2004; reviewed by Vennekens et al., 2002). Thus, divalent cation selectivity was utilized in order to further characterize the LPA-mediated Ca^{2+}

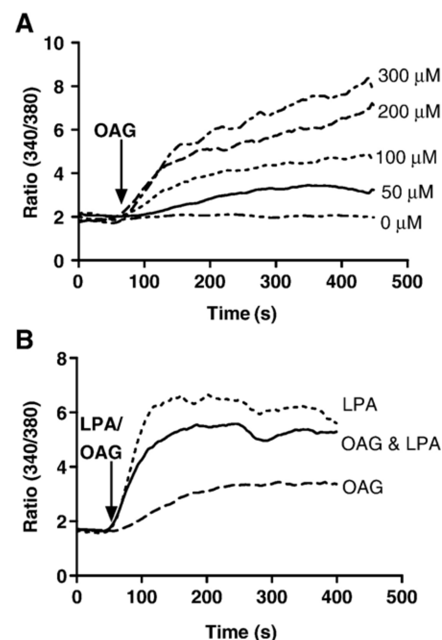

Fig. 2. OAG and LPA stimulated Ca^{2+} mobilization of BLCLs. BLCLs were loaded with 1 μM fura-2. A. OAG (0–300 μM) applied to one BLCL (1×10^6 cells/mL) ($n=3$ determinations at each concentration). B. LPA (100 μM), OAG (100 μM) or both were added simultaneously to buffer containing Ca^{2+} (1 mM). The combination of LPA and OAG resulted in a significantly greater Δ (see Materials and methods) response compared with OAG alone ($F=149$, $df=2$, 10, $p<0.001$; Tukey $p<0.001$) but smaller than LPA stimulated Δ responses (Tukey, $p=0.001$) ($n=4$–5 for each comparison condition).

responses in this cell model, similar to other cellular investigations (Gamberucci et al., 2002; Hassock et al., 2002; Trebak et al., 2003; Venkatachalam et al., 2002; Zeng et al., 2004).

To establish that OAG mediates non-selective cation entry through TRPC-like channels in BLCLs, OAG stimulation of BLCLs was carried out following re-addition of either 1 mM Ba^{2+} or Ca^{2+} to the extracellular Ca^{2+}-free buffer. Stimulation with 100 μM OAG elicited Ba^{2+} entry of a magnitude similar to that of Ca^{2+} entry ($t=0.13$, $df=9$, $p=0.898$) (Fig. 3A). In contrast, treatment of BLCLs in Ca^{2+}-free medium with TG, which induces transient depletion of ER Ca^{2+} stores and SOC entry, caused a substantial increase in $[Ca^{2+}]_i$ upon re-addition of Ca^{2+} to the extracellular media, but only a small magnitude of Ba^{2+} influx ($F=71$, $df=2,10$, $p<0.001$) compared with the former response (Tukey $p<0.001$; Fig. 3B). As expected, there was no cation influx observed in the absence of Ba^{2+} or Ca^{2+} in the buffer.

To estimate the magnitude of the TRPC-like component of the LPA response, the maximal stimulating concentration of LPA (100 μM) used in previous studies demonstrating Ca^{2+} abnormalities in BLCLs from BD subjects (Wasserman et al., 2004), was applied to dye-loaded BLCLs in extracellular buffer containing either Ca^{2+} or Ba^{2+}. The resultant Ca^{2+} entry showed the typical rapid influx and high fluorescence ratio observed after LPA stimulation (Fig. 3C) (Wasserman et al., 2004). In the absence of Ca^{2+} or Ba^{2+} in the medium, there was a small and delayed increase in fluorescence signal that likely derives from the release of Ca^{2+} from intracellular storage pools. However, the rate of rise of the Ca^{2+} concentration was slow (beginning after approximately after 100 s) compared to what would be expected for an IP_3 mediated store depletion (occurring in less than 1 s) of the ER (Hattori et al., 2004; O'Neill et al., 2002). Therefore, it appears that LPA predominantly mobilizes intracellular Ca^{2+} via an IP_3-independent pathway in the BLCL model. Interestingly, the Ba^{2+} influx response was significantly larger than the increases obtained in the Ca^{2+}/Ba^{2+}-free medium (Fig. 3C) ($F=89$, $df=2,16$, $p<0.001$; Tukey $p=0.004$) and was of a similar magnitude to the maximum Ba^{2+} influx observed in response to stimulation with 100 μM OAG ($t=0.83$, $df=11$, $p=0.425$) (Fig. 3A).

Next, the non-selective PLC activator, *m*-3M3FBS, was used to examine the possibility that LPA-mediated Ba^{2+} influx is secondary to PLC activation and diacylglycerol (DAG) production, which then may act on TRPC3 (Hofmann et al., 1999). This agent has been shown to activate PI-dependent Ca^{2+} mobilization in a number of different cell lines (Bae et al., 2003). In BLCLs, *m*-3M3FBS stimulated Ca^{2+} mobilization in a concentration-dependent manner with a maximal stimulating concentration of 50 μM (data not shown). With Ca^{2+} in the extracellular buffer, application of *m*-3M3FBS elicited an

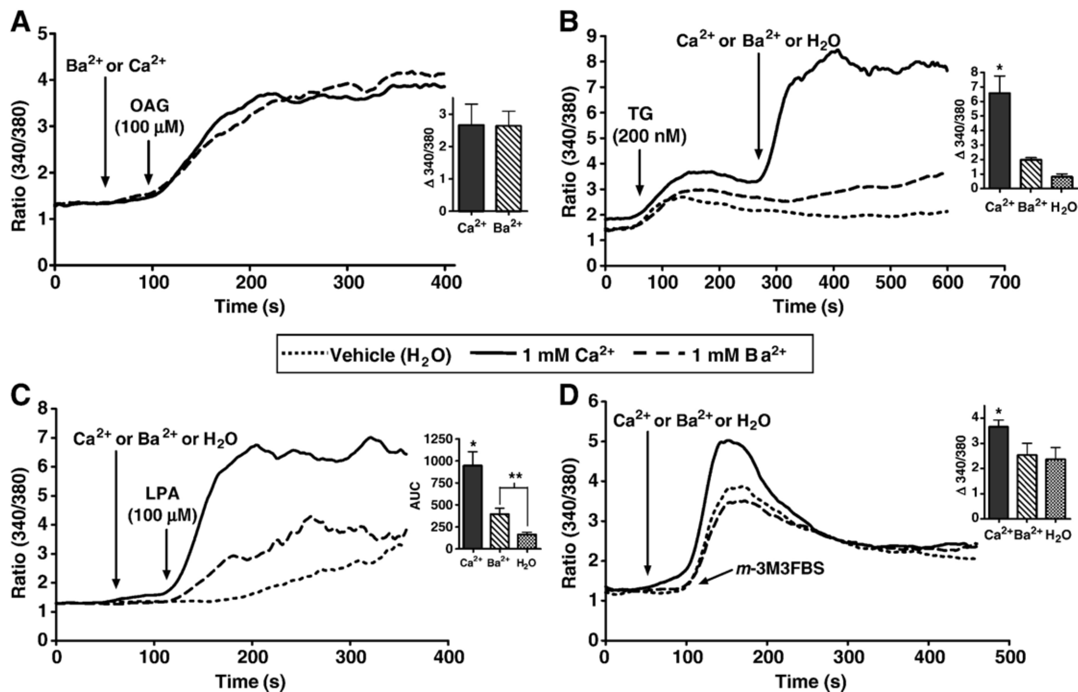

Fig. 3. Effects of LPA, OAG and *m*-3M3FBS on divalent cation entry in BLCLs. BLCLs (1×10^6 cells/mL) were loaded with fura-2 (1 μM) in Ca^{2+} free buffer. A. OAG (100 μM) stimulated 1 mM Ba^{2+} or Ca^{2+} influx to the same Δ magnitude ($t=0.13$, $df=9$, $p=0.898$). Representative response curves are shown. B. Thapsigargin (TG)-induced Ba^{2+} entry was examined. Three minutes after TG (200 nM), Ba^{2+} (1 mM), Ca^{2+} (1 mM) or an equivalent volume of vehicle (H_2O) was added to the extracellular buffer. The Δ magnitude of Ba^{2+} entry was significantly smaller than that of Ca^{2+} ($F=71$, $df=2,10$, $p<0.001$; Tukey $p<0.001$). C. Ca^{2+} (1 mM), Ba^{2+} (1 mM) or vehicle (H_2O, 20 μL) was added after 1 min of baseline signal recording, followed 1 min later by LPA (100 μM). Ba^{2+} influx was significantly greater than vehicle ($F=89$, $df=2,16$, $p<0.001$; Tukey $p=0.004$) and was of a similar Δ magnitude to that observed following stimulation with OAG (100 μM) ($t=0.83$, $df=11$, $p=0.425$). A, B and C: $n=5$ for each cation, 3 for H_2O. D. Ca^{2+} (1 mM), Ba^{2+} (1 mM) or vehicle (H_2O, 20 μL) was added after 1 min of baseline signal recording, followed 1 min later by *m*-3M3FBS (50 μM). The ΔCa^{2+} influx was significantly greater than controls ($F=17$, $df=2$, 15, $p<0.001$; Tukey $p<0.001$), while no Ba^{2+} entry was observed (Tukey $p=0.782$). $n=6$ for each comparison condition; representative curves shown.

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increase in fluorescence ratios that were significantly greater than those observed in Ca^{2+}/Ba^{2+}-free medium ($F=17$, $df=2$, 15, $p<0.001$; Tukey $p<0.001$). In contrast, *m*-3M3FBS increased fura-2 ratios in Ba^{2+}-containing medium to a value similar to that observed in cells incubated in medium that was free of Ca^{2+} and Ba^{2+} (Tukey $p=0.782$) (Fig. 3D), indicating that no significant Ba^{2+} influx occurred.

Effects of pertussis toxin and PLC inhibition on LPA stimulated Ca^{2+} responses

To determine what portion of the LPA-stimulated Ca^{2+} response is mediated through GPCR and in turn PLC activation, the effect of treatments with inhibitors of $G_{i/o}$ (PTX) and PLC (U-73122) were examined on LPA-provoked Ca^{2+} mobilization. Pretreatment of cells with PTX inhibited LPA-stimulated Ca^{2+} responses from 10% to 50% (data not shown) across a number of cell lines from individual subjects. In addition, U-73122 did not appear to attenuate LPA-mediated Ca^{2+} mobilization in the BLCL model (Fig. 4A). Under the same experimental conditions, U-73122 fully inhibited bradykinin (200 nM)-stimulated Ca^{2+} responses in PC12 cells (Fig. 4B), thus validating the methodology and efficacy of the inhibitor preparation.

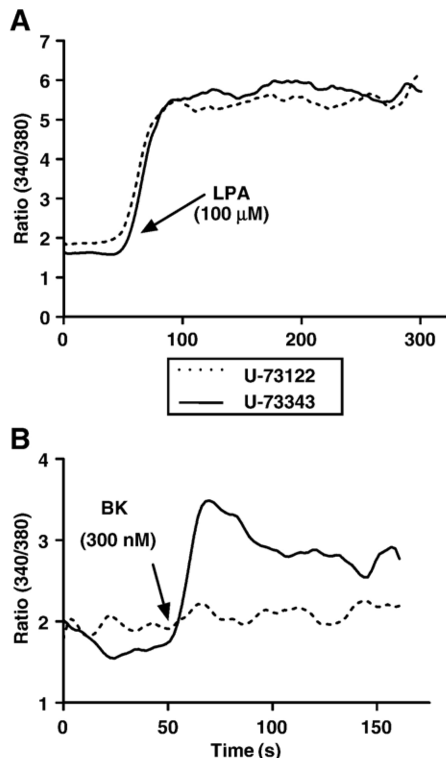

Fig. 4. Effect of PLC inhibitor on agonist-induced Ca^{2+} mobilization in BLCLs and PC12 cells. A. BLCLs (1×10^6 cells/mL) were loaded with fura-2 (1 μM). LPA (100 μM)-stimulated Ca^{2+} response was not significantly inhibited by pretreatment with U-73122 in comparison with the inactive control, U-73343. Representative curve shown for each condition ($n=10$). B. PC12 cells (1×10^6 cells/mL) were loaded with fura-2 (2 μM). BK (300 nM)-stimulated Ca^{2+} response was completely abrogated in cells pre-treated with U-73122 (active PLC inhibitor) in comparison to those exposed to U-73343 (the inactive control). Representative response curves are shown ($n=5$).

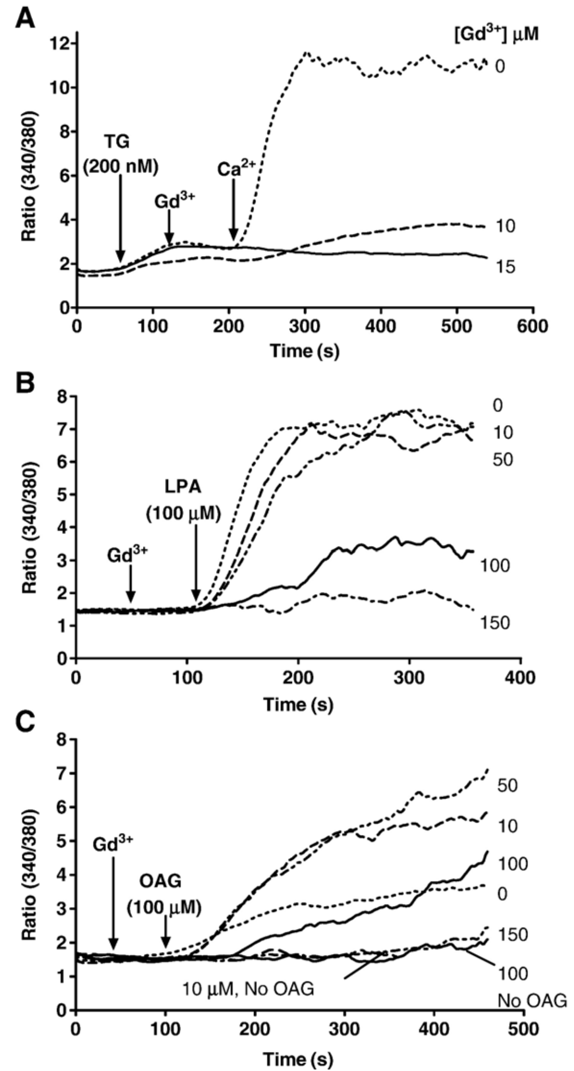

Fig. 5. Effects of Gd^{3+} on TG, LPA and OAG stimulated Ca^{2+} responses. A. Gd^{3+} (5–15 μM) completely abolished the influx of Ca^{2+} triggered by TG in BLCLs. B. Gd^{3+} (5–15 μM) minimally inhibited the Δ LPA (100 μM) stimulated Ca^{2+} responses; Gd^{3+} (100 μM) resulted in approximately 50% reduction in the LPA response. C. The ΔCa^{2+} signal in response to OAG (100 μM) was potentiated by Gd^{3+} (10–50 μM). The magnitude of the ΔCa^{2+} influx stimulated by OAG and LPA was similar at 100 μM Gd^{3+} ($t=0.31$, $df=4$, $p=0.773$), 50 μM ($t=0.73$, $df=4$, $p=0.505$), and 10 μM ($t=0.546$, $df=4$, $p=0.614$). All frames: $n=3$ separate determinations at each Gd^{3+} concentration; representative curves shown.

Effect of gadolinium on LPA and OAG stimulated Ca^{2+} responses

Gadolinium (Gd^{3+}) inhibits capacitative cation entry at low micromolar concentrations (Jung et al., 2003; Lievremont et al., 2004; Shlykov et al., 2003; Trebak et al., 2003), whereas cation influx mediated by TRPC3/6/7 is insensitive to low Gd^{3+} (~10 μM) concentrations (Lievremont et al., 2004; Okada et al., 1999; Trebak et al., 2003; Zhu et al., 1998). Thus, the use of Gd^{3+} should inhibit LPA-induced Ca^{2+} entry through SOC channels while permitting the measurement of TRPC mediated Ca^{2+} entry (Lievremont et al., 2004; Okada et al., 1999; Trebak et al., 2003; Zhu et al., 1998). Consonant with these known effects,

10–15 μM Gd^{3+} completely abolished the influx of extracellular Ca^{2+} triggered by TG in BLCLs (Fig. 5A). In marked contrast, significant inhibition of LPA-stimulated Ca^{2+} response was not evident until 100 μM Gd^{3+} and this concentration was not sufficient to completely inhibit the response (Fig. 5B). Furthermore, OAG (100 μM)-induced Ca^{2+} entry was markedly potentiated by 10–50 μM Gd^{3+}, but not 100 μM Gd^{3+} (Fig. 5C). In fact, the magnitude of the ΔCa^{2+} influx as measured by the maximum peak height stimulated by OAG (100 μM) and LPA (100 μM) was similar at three Gd^{3+} concentrations measured [100 μM Gd^{3+} (t=0.31, df=4, p=0.773), 50 μM (t=0.73, df=4, p=0.505), and 10 μM (t=0.546, df=4, p=0.614)].

Discussion

The principal finding of this study is that 100 μM LPA stimulates Ca^{2+} mobilization in BLCLs, at least in part, through a TRPC3-like channel. This inference is supported by seven principal sets of observations. First, OAG activated both Ca^{2+} and Ba^{2+} mobilization in BLCLs confirming the lack of cation selectivity, characteristic of TRPC subtypes in general (Hofmann et al., 1999; Trebak et al., 2003; Zhu et al., 1998). Secondly, among the known subtypes of TRPCs expressed in various tissues and cell types, only TRPC1, 3 and 5 like immunoreactivities were detected in BLCLs, implicating TRPC3 as the likely candidate for OAG-provoked Ca^{2+} responses in this cell model (Andreopoulos et al., 2004). Third, LPA activates non-selective cation entry as demonstrated by the significant magnitude of LPA-induced Ba^{2+} entry, similar in extent to that observed following OAG stimulation. Fourth, stimulation with *m*-3M3FBS did not result in Ba^{2+} influx, implying that PLC activation under these conditions does not produce sufficient intracellular DAG to activate TRPC channels that respond to this second messenger. Fifth, inhibition of $G_{i/o}$ and PLC did not result in reproducible attenuation of the LPA response, as would be expected if this agent stimulated Ca^{2+} mobilization primarily through a GPCR mode of activation. Sixth, simultaneous addition of OAG and LPA resulted in a reduced net Ca^{2+} influx rather than an additive response, suggesting that these agents activate a common target. Finally, the similar magnitudes in OAG and LPA-stimulated ΔCa^{2+} responses measured in the presence of the SOC inhibitor, Gd^{3+}, suggests that these agents act on the same signalling pathways.

Stimulation with LPA, conducted in the presence of divalent cations, Ba^{2+} or Ca^{2+}, triggered two types of response patterns: with Ca^{2+} in the buffer there was a rapid rise in the response to a magnitude of approximately 7 fluorescence ratio units, while with Ba^{2+} substituted for Ca^{2+} there was a slower rate of influx and a maximal response of approximately 3.5 fluorescence ratio units (Fig. 3C). This differential response in the presence of Ca^{2+} as compared with Ba^{2+} suggests that LPA may act via two types of channels, one of which exhibits a greater degree of Ca^{2+} selectivity. In this regard, the greater response observed in the presence of Ca^{2+} likely represents the combination of Ca^{2+} selective SOC activation and non-selective cation (Ba^{2+}/Ca^{2+}) permeable receptor-mediated TRPC channel gating. Furthermore, the magnitude of Ba^{2+} entry elicited by LPA stimulation (Fig. 3C) was similar to that of OAG-mediated cation entry (Fig. 3A), suggesting that LPA-induced Ba^{2+} influx occurs via TRPC3-like channel gating.

Consonant with the findings in platelets (Hassock et al., 2002), depletion of ER Ca^{2+} store by TG activated cation channels that are Ca^{2+} selective, with little Ba^{2+} entry observed in BLCLs (Fig. 3B). Therefore, the influx of Ba^{2+} in this cell model reports both receptor-operated second messenger-mediated and/or direct channel activation but not SOC-mediated influx. The very small magnitude of Ba^{2+} influx observed following TG-induced SOC activation suggests that TRPC channels in the BLCL model are predominantly ROC regulated. This is noteworthy as the mode of regulation of TRPC channels is controversial, with many studies conducted in expression systems that may lack the proper stoichiometry of accessory proteins required for "true" physiological functionality (Clapham et al., 2001; Vazquez et al., 2004; Vennekens et al., 2002).

Further examination of divalent cation selectivity in BLCLs demonstrated that PLC stimulation gates Ca^{2+} but not Ba^{2+} permeable channels, likely due to the activation of Ca^{2+} selective SOC channels. This result suggests that the extent of endogenous DAG produced in response to direct activation of PLC, under our experimental conditions, is insufficient to activate those non-selective TRPC channels responsive to this second messenger, strengthening the hypothesis that Ba^{2+} entry during LPA stimulation is not due to intracellular DAG accumulation but rather the direct activation of these channels. However, the use of *m*-3M3FBS as a PLC stimulator has become contentious as questions concerning its specificity have been recently raised (Krjukova et al., 2004).

It has been shown that LPA stimulates GPCRs leading to PLC activation (Anliker and Chun, 2004) and BLCLs express LPA receptor subtypes 1–3 as confirmed using RT-PCR (data not shown). Experiments involving the inhibition of PLC and $G_{i/o}$ were used to elucidate the magnitude of the LPA response dependent on a GPCR mode of activation. Indeed, Rosskopf et al. (1998) showed that LPA-stimulated Ca^{2+} mobilization in BLCLs reached a plateau between 1–10 μM which was almost completely inhibited by pre-treatment with PTX (50 ng/mL, 16 h), but the fold response was very small (1×) in contrast to that obtained (11–15×) upon extension of the stimulating concentration into the high μM range (Wasserman et al., 2004). The fact that LPA-mediated Ca^{2+} responses were only partially PTX-sensitive (200 ng/mL, 24 h) and minimally affected by PLC inhibition, supports the notion that a major fraction of the Ca^{2+} response produced by 100 μM LPA involves a non-GPCR component. Of note, such lack of inhibition as observed in this work has been reported by others examining the mode of action of LPA (Itagaki et al., 2005; Lee et al., 2004), adding further credence to the notion that LPA agonism occurs via multiple pathways. One impediment to further elucidation of the suspected action of LPA on TRPC3-like channels is the lack of current knowledge regarding the specifics of OAG-mediated TRPC3 activation and TRPC regulation in general.

The trivalent cation, Gd^{3+}, has been described as a capacitative channel inhibitor at low micromolar concentrations (Jung et al., 2003; Lievremont et al., 2004; Shlykov et al., 2003;

Trebak et al., 2003). Some studies report inhibition of TRPC3 channels upon Gd^{3+} addition in the low micromolar range (Gamberucci et al., 2002; Halaszovich et al., 2000; Kamouchi et al., 1999; Shlykov et al., 2003), while others have described the TRPC3 subfamily of channels as insensitive to low micromolar Gd^{3+} (Lievremont et al., 2005, 2004; Okada et al., 1999; Trebak et al., 2003; Zhu et al., 1998). The differential sensitivity of TRPC3 to Gd^{3+} appears to be dependent on its mode of regulation, as receptor-activated cation entry through TRPC3 channels in HEK293 cells was not affected by Gd^{3+}, whereas store-operated responses elicited in DT40 cells were completely blocked (Trebak et al., 2002). Consistent with this idea, we showed that TG-induced Ca^{2+} entry was blocked by 10–15 μM Gd^{3+}. On the other hand, the Ca^{2+} influx induced by OAG was significantly potentiated in the presence of 10–50 μM, but not 100 μM, of Gd^{3+} in BLCLs (Fig. 5C). Interestingly, potentiation of Ca^{2+} mobilization responses by Gd^{3+} has been observed in cells overexpressing TRPC4 and 5 channels (Jung et al., 2003; Zeng et al., 2004). However, if it were that such an effect of Gd^{3+} on TRPC4/5 mediated Ca^{2+} influx also occurs in BLCLs, then one would expect Gd^{3+}, in the absence of OAG, to activate Ca^{2+} influx. In contrast, addition of 10 or 100 μM Gd^{3+} alone to BLCL preparations did not result in any change in the fura-2 ratios (Fig. 5C). Regardless of the mechanism involved, it is interesting to note that the net Δ magnitude of LPA-induced Ca^{2+} responses at 10, 50 and 100 μM Gd^{3+}, were similar to OAG-activated Ca^{2+} influx in the presence of corresponding Gd^{3+} concentrations (see Fig. 5B and C). Since OAG activates TRPC3 but not TRPC1 and 5 (Hofmann et al., 1999), it is likely that LPA activates only TRPC3 in this cell model under these experimental conditions. Otherwise, one would expect the response to LPA to exceed that of OAG, if additional channels were being affected. Of note, different rates of Ca^{2+} influx were exhibited by OAG and LPA activation in the presence of Gd^{3+}, possibly due to dissimilar membrane permeation, tertiary structure, or other characteristics between these two compounds that would govern accessibility of the drug to the site of action.

Recently, Flemming et al. (2006) described an apparent direct activation of TRPC5 channels by a lysophospholipid (lysophosphatidylcholine, LPC). However, no Ca^{2+} mobilization was induced by LPC (10–100 μM) in the BLCL model (Wasserman, 2003). Perhaps this is due to cell specific differences such as dissimilar TRPC subunit composition. However, the report of a GPCR-independent activation of the TRPC channel family by LPC (Flemming et al., 2006), in conjunction with the findings presented here, supports the notion that lysophospholipid-mediated signalling is an additional mechanism by which TRPC channels may be regulated.

Conclusions

Collectively, the above findings support the notion that LPA induces Ca^{2+} mobilization, in part, through a TRPC3-like channel in human BLCLs. This inference has particularly important implications for the interpretation of the altered LPA-stimulated Ca^{2+} mobilization that has been observed in BLCLs from patients with BD compared with those from healthy subjects, and the effect of chronic lithium treatment of BLCLs to attenuate LPA-stimulated Ca^{2+} responses (Wasserman et al., 2004). The conclusions of this report and that of others regarding the activation profile of LPA highlight the complexity of the response triggered by this agent, bring the suitability of LPA as a probe for signal transduction abnormalities into question. Perhaps, an array of more specific probes, such as OAG, TG and the PLC activator (assuming its mode of action is substantiated), would provide more detailed information regarding the nature of putative pathophysiological disturbances affecting intracellular Ca^{2+} dynamics in disease such as bipolar disorder.

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References

Andreopoulos, S., Wasserman, M., Woo, K., Li, P., Warsh, J., 2004. Chronic lithium treatment of B lymphoblasts from bipolar disorder patients reduces transient receptor potential channel 3 levels. The Pharmacogenomics Journal 4 (6), 365–373.

Anliker, B., Chun, J., 2004. Cell surface receptors in lysophospholipid signaling. Seminars in Cell and Developmental Biology 15 (5), 457–465.

Bae, Y.-S., Lee, T.G., Park, J.C., Hur, J.H., Kim, Y., Heo, K., Kwak, J.-Y., Suh, P.-G., Ryu, S.H., 2003. Identification of a compound that directly stimulates phospholipase C activity. Molecular Pharmacology 63 (5), 1043–1050.

Clapham, D.E., Runnels, L.W., Strubing, C., 2001. The TRP ion channel family. Nature Reviews. Neuroscience 2 (6), 387–396.

Dubovsky, S.L., Christiano, J., Daniell, L.C., Franks, R.D., Murphy, J., Adler, L., Baker, N., Harris, A., 1989. Increased platelet intracellular calcium concentration in patients with bipolar affective disorders. Archives of General Psychiatry 46 (7), 632–638.

Emamghoreishi, M., Schlichter, L., Li, P.P., Parikh, S., Sen, J., Kamble, A., Warsh, J.J., 1997. High intracellular calcium concentrations in transformed lymphoblasts from subjects with bipolar I disorder. American Journal of Psychiatry 154 (7), 976–982.

Flemming, P.K., Dedman, A.M., Xu, S.Z., Li, J., Zeng, F., Naylor, J., Benham, C. D., Bateson, A.N., Muraki, K., Beech, D.J., 2006. Sensing of lysophospholipids by TRPC5 calcium channel. Journal of Biological Chemistry 281 (8), 4977–4982.

Flockerzi, V., Jung, C., Aberle, T., Meissner, M., Freichel, M., Philipp, S., Nastainczyk, W., Maurer, P., Zimmermann, R., 2005. Specific detection and semi-quantitative analysis of TRPC4 protein expression by antibodies. Pflugers Archiv European Journal of Physiology 451 (1), 81–86.

Gamberucci, A., Giursato, E., Pizzo, P., Tassi, M., Giunti, R., McIntosh, D.P., Benedetti, A., 2002. Diacylglycerol activates the influx of extracellular cations in T-lymphocytes independently of intracellular calcium-store depletion and possibly involving endogenous TRP6 gene products. Biochemical Journal 364 (Pt 1), 245–254.

Hahn, C.-G., Gomez, G., Restrepo, D., Friedman, E., Josiassen, R., Pribitkin, E.A., Lowry, L.D., Gallop, R.J., Rawson, N.E., 2005. Aberrant intracellular calcium signaling in olfactory neurons from patients with bipolar disorder. American Journal of Psychiatry 162 (3), 616–618.

Halaszovich, C.R., Zitt, C., Jungling, E., Luckhoff, A., 2000. Inhibition of TRP3 channels by lanthanides. Block from the cytosolic side of the plasma membrane. Journal of Biological Chemistry 275 (48), 37423–37428.

Hassock, S.R., Zhu, M.X., Trost, C., Flockerzi, V., Authi, K.S., 2002. Expression and role of TRPC proteins in human platelets: evidence that TRP6 forms the store-independent calcium entry channel. Blood 100 (8), 2801–2811.

Hattori, M., Suzuki, A.Z., Higo, T., Miyauchi, H., Michikawa, T., Nakamura, T., Inoue, T., Mikoshiba, K., 2004. Distinct roles of Inositol 1,4,5-trisphosphate receptor types 1 and 3 in Ca^{2+} signaling. Journal of Biological Chemistry 279 (12), 11967–11975.

Hofmann, T., Obukhov, A.G., Schaefer, M., Harteneck, C., Gudermann, T., Schultz, G., 1999. Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol. Nature 397 (6716), 259–263.

Hofmann, T., Schaefer, M., Schultz, G., Gudermann, T., 2000. Transient receptor potential channels as molecular substrates of receptor-mediated cation entry. Journal of Molecular Medicine 78 (1), 14–25.

Hofmann, T., Schaefer, M., Schultz, G., Gudermann, T., 2002. Subunit composition of mammalian transient receptor potential channels in living cells. Proceedings of the National Academy of Sciences of the United States of America 99 (11), 7461–7466.

Hoth, M., Penner, R., 1992. Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature 355 (6358), 353–356.

Itagaki, K., Kannan, K.B., Hauser, C.J., 2005. Lysophosphatidic acid triggers calcium entry through a non-store-operated pathways in human neutrophils. Journal of Leukocyte Biology 77 (2), 181–189.

Jung, S., Muhle, A., Schaefer, M., Strotmann, R., Schultz, G., Plant, T.D., 2003. Lanthanides potentiate TRPC5 currents by an action at extracellular sites close to the pore mouth. Journal of Biological Chemistry 278 (6), 3562–3571.

Kamouchi, M., Philipp, S., Flockerzi, V., Wissenbach, U., Mamin, A., Raeymaekers, L., Eggermont, J., Droogmans, G., Nilius, B., 1999. Properties of heterologously expressed hTRP3 channels in bovine pulmonary artery endothelial cells. Journal of Physiology 518 (2), 345–358.

Krjukova, J., Holmqvist, T., Danis, A.S., Akerman, K.E.O., Kukkonen, J.P., 2004. Phospholipase C activator *m*-3M3FBS affects Ca^{2+} homeostasis independently of phospholipase C activation. British Journal of Pharmacology 143 (1), 3–7.

Lee, H.-Y., Kang, H.-K., Yoon, H.-R., Kwak, J.-Y., Bae, Y.-S., 2004. Lysophosphatidic acid is a mediator of Trp-Lys-Tyr-Met-Val-D-Met-induced calcium influx. Biochemical and Biophysical Research Communications 324 (1), 458–465.

Lievremont, J.-P., Bird, G.S.J., James, W., Putney, J., 2004. Canonical transient receptor potential TRPC7 can function both as a receptor-and store-operated channel in HEK293 cells. American Journal of Physiology. Cell Physiology 287 (6), C1709–C1716.

Lievremont, J.-P., Bird, G.S., Putney Jr., J.W., 2005. Mechanism of inhibition of TRPC cation channels by 2-aminoethoxydiphenylborane. Molecular Pharmacology 68 (3), 758–762.

McKay, R.R., Szymeczek-Seay, C.L., Lievremont, J.-P., Bird, G.S.J., Zitt, C., Jungling, E., Luckoff, A., Putney, J.J.W., 2000. Cloning and expression of the human transient receptor potential 4 (TRP4) gene: localization and functional expression of human TRP4 and TRP3. Biochemical Journal 351 (Pt 3), 735–746.

Ohta, H., Sato, K., Murata, N., Damirin, A., Malchinkhuu, E., Kon, J., Kimura, T., Tobo, M., Yamazaki, Y., Watanabe, T., Yagi, M., Sato, M., Suzuki, R., Murooka, H., Sakai, T., Nishitoba, T., Im, D.-S., Nochi, H., Tamoto, K., Tomura, H., Okajima, F., 2003. Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors. Molecular Pharmacology 64 (4), 994–1005.

Okada, T., Inoue, R., Yamazaki, K., Maeda, A., Kurosaki, T., Yamakuni, T., Tanaka, I., Shimizu, S., Ikenaka, K., Imoto, K., Mori, Y., 1999. Molecular and functional characterization of a novel mouse transient receptor potential protein homologue TRP7. $Ca^{(2+)}$-permeable cation channel that is constitutively activated and enhanced by stimulation of G protein-coupled receptor. Journal of Biological Chemistry 274 (39), 27359–27370.

O'Neill, A.F., Hagar, R.E., Zipfel, W.R., Nathanson, M.H., Ehrlich, B.E., 2002. Regulation of the type III InsP3 receptor by InsP3 and calcium. Biochemical and Biophysical Research Communications 294 (3), 719–725.

Riccio, A., Mattei, C., Kelsell, R.E., Medhurst, A.D., Calver, A.R., Randall, A.D., Davis, J.B., Benham, C.D., Pangalos, M.N., 2002. Cloning and functional expression of human short TRP7, a candidate protein for store-operated Ca^{2+} influx. Journal of Biological Chemistry 277 (14), 12302–12309.

Rosskopf, D., Daelman, W., Busch, S., Schurks, M., Hartung, K., Kribben, A., Michel, M.C., Siffert, W., 1998. Growth factor-like action of lysophospatidic acid on human B lymphoblasts. American Journal of Physiology 274 (6 Pt 1), C1573–C1582.

Segura, B.J., Zhang, W., Cowles, R.A., Xiao, L., Lin, T.R., Logsdon, C., Mulholland, M.W., 2004. Lysophosphatidic acid stimulates calcium transients in enteric glia. Neuroscience 123 (3), 687–693.

Shlykov, S.G., Yang, M., Alcorn, J.L., Sanborn, B.M., 2003. Capacitative cation entry in human myometrial cells and augmentation by hTRPC3 overexpression. Biology of Reproduction 69 (2), 647–655.

Sossey-Alaoui, K., Lyon, J.A., Jones, L., Abidi, F.E., Hartung, A.J., Hane, B., Schwartz, C.E., Stevenson, R.E., Srivastava, A.K., 1999. Molecular cloning and characterization of TRPC5 (HTRP5), the human homologue of a mouse brain receptor-activated capacitative Ca^{2+} entry channel. Genomics 60 (3), 330–340.

Thebault, S., Zholos, A., Enfissi, A., Slomianny, C., Dewailly, E., Roudbaraki, M., Parys, J., Prevarskaya, N., 2005. Receptor-operated Ca^{2+} entry mediated by TRPC3/TRPC6 proteins in rat prostate smooth muscle (PS1) cell line. Journal of Cellular Physiology 204 (1), 320–328.

Trebak, M., St. J. Bird, G., McKay, R.R., Putney Jr., J.W., 2002. Comparison of human TRPC3 channels in receptor-activated and store-operated modes. Differential sensitivity to channel blockers suggests fundamental differences in channel composition. Journal of Biological Chemistry 277 (24), 21617–21623.

Trebak, M., Vazquez, G., Bird, G.S.J., James, W., Putney, J., 2003. The TRPC3/6/7 subfamily of cation channels. Cell Calcium 33 (5−6), 451–461.

Vazquez, G., Wedel, B.J., Aziz, O., Trebak, M., James, W., Putney, J., 2004. The mammalian TRPC cation channels. Biochimica et Biophysica Acta 1742 (1−3), 21–36.

Venkatachalam, K., Rossum, D.B.v., Petterson, R.L., Ma, H.-T., Gill, D.L., 2002. The cellular and molecular basis of store-operated calcium entry. Nature Cell Biology 4 (11), E263–E272.

Vennekens, R., Voets, T., Bindels, R.J.M., Droogmans, G., Nilius, B., 2002. Current understanding of mammalian TRP homologues. Cell Calcium 31 (6), 253–264.

Warsh, J.J., Andreopoulos, S., Li, P.P., 2004. Role of intracellular calcium signaling in the pathophysiology and pharmacotherapy of bipolar disorder: current status. Clinical Neuroscience Research 4, 201–213.

Wasserman, M., 2003. Abnormal intracellular calcium mobilization and modulation by chronic lithium treatment in patients with bipolar disorder. A thesis of the Department of Pharmacology. University of Toronto, Toronto, pp. 101–102.

Wasserman, M.J., Corson, T.W., Sibony, D., Cooke, R.G., Parikh, S.V., Pennefather, P.S., Li, P.P., Warsh, J.J., 2004. Chronic lithium treatment attenuates intracellular calcium mobilization. Neuropsychopharmacology 29 (4), 759–769.

Zeng, F., Xu, S.-Z., Jackson, P.K., McHugh, D., Kumar, N., Fountain, S.J., Beech, D.J., 2004. Human TRPC5 channel activated by a multiplicity of signals in a single cell. Journal of Physiology 559 (Pt 3), 739–750.

Zhu, X., Jiang, M., Birnbaumer, L., 1998. Receptor-activated Ca^{2+} influx via human Trp3 stably expressed in human embryonic kidney (HEK)293 cells. Journal of Biological Chemistry 273 (1), 133–142.