BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 161, 308–320

0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

GPR55-dependent and -independent ion signalling in response to lysophosphatidylinositol in endothelial cells

Alexander Bondarenko, Markus Waldeck-Weiermair, Shamim Naghdi, Michael Poteser, Roland Malli and Wolfgang F Graier

Institute of Molecular Biology and Biochemistry, Center of Molecular Medicine, Medical University of Graz, Graz, Austria

Background and purpose: The glycerol-based lysophospholipid lysophosphatidylinositol (LPI) is an endogenous agonist of the G-protein-coupled receptor 55 (GPR55) exhibiting cannabinoid receptor-like properties in endothelial cells. To estimate the contribution of GPR55 to the physiological effects of LPI, the GPR55-dependent and -independent electrical responses in this cell type were investigated.

Experimental approach: Applying small interference RNA-mediated knock-down and transient overexpression, GPR55-dependent and -independent effects of LPI on cytosolic free Ca^{2+} concentration, membrane potential and transmembrane ion currents were studied in EA.hy296 cells.

Key results: In a GPR55-dependent, GDPβS and U73122-sensitive manner, LPI induced rapid and transient intracellular Ca^{2+} release that was associated with activation of charybdotoxin–sensitive, large conductance, Ca^{2+}-activated, K^+ channels (BK_{Ca}) and temporary membrane hyperpolarization. Following these initial electrical reactions, LPI elicited GPR55-independent long-lasting Na^+ loading and a non-selective inward current causing sustained membrane depolarization that depended on extracellular Ca^{2+} and Na^+ and was partially inhibited by Ni^{2+} and La^{3+}. This inward current was due to the activation of a voltage-independent non-selective cation current. The Ni^{2+} and La^{3+}-insensitive depolarization with LPI was prevented by inhibition of the Na/K-ATPase by ouabain.

Conclusions and implications: LPI elicited a biphasic response in endothelial cells of which the immediate Ca^{2+} signalling depends on GPR55 while the subsequent depolarization is due to Na^+ loading via non-selective cation channels and an inhibition of the Na/K-ATPase. Thus, LPI is a potent signalling molecule that affects endothelial functions by modulating several cellular electrical responses that are only partially linked to GPR55.

British Journal of Pharmacology (2010) **161,** 308–320; doi:10.1111/j.1476-5381.2010.00744.x

Keywords: endothelial cells; GPR55; lysophospholipid signalling; membrane potential; non-selective cation channel; patch-clamp; rimonabant

Abbreviations: BK_{Ca}, large conductance, Ca^{2+} activated, K^+ channels; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid disodium salt hydrate; DMEM, Dulbecco's modified Eagle's medium; EDHF, endothelium-derived hyperpolarizing factor; GDPβS, guanosine 5′-O-(2-thiodiphosphate); GPCR, G-protein-coupled receptor; IP_3, inositol 1,4,5-trisphosphate; KAsp, potassium aspartate; LP, lysophospholipid; LPI, lysophosphatidylinositol; SOCE, store-operated Ca^{2+} entry; TRPV1, transient receptor potential cation channel V1

Introduction

Lysophospholipids (LPs) have been recognized as potent signalling molecules that modulate many cell functions in a variety of tissues including the cardiovascular, immune, nervous, and reproductive systems (Gardell *et al*., 2006). The generation of these signalling molecules is most often linked to the metabolism of membrane phospholipids by enzymes that are located at the inner side of the plasma membrane and are activated upon cell stimulation. Initially, LPs have thus been discussed to serve as second-messenger molecules that modulate intracellular signalling events (Corda *et al*., 2002). However, recent findings point to LPs also as intercellular signalling molecules that are involved in cell-to-cell communication. This assumption is based on reports that LPs are transported to, and even produced in, the extracellular

Correspondence: Wolfgang F Graier, Molecular and Cellular Physiology Research Unit, Institute of Molecular Biology and Biochemistry, Center of Molecular Medicine, Medical University of Graz, Harrachgasse 21/III, 8010 Graz, Austria. E-mail: wolfgang.graier@medunigraz.at

Received 4 January 2010; revised 31 January 2010; accepted 6 February 2010

space and that cell surface receptors that are specifically activated by certain LPs have been identified.

However, the diverse effects of different LPs as signalling molecules are only marginally explored on the molecular level, although they deserve particular interest in order to understand the molecular processes of lipotoxic pathways. Increased levels of LPs have been found particularly under pathological conditions in the vasculature, such as atherosclerosis (Ridgway *et al*., 1999), and hypertension (Smyth *et al*., 2008), indicating that LPs are also important signalling molecules under pathological condition (Gardell *et al*., 2006). Accordingly, the development of pharmacological tools that interfere with receptor-mediated LP signalling is nowadays thought to represent a promising approach for the development of novel therapeutic strategies against vascular diseases (Gardell *et al*., 2006).

One of the less examined LPs is lysophosphatidylinositol (LPI), a glycerol-based LP that has recently been reported to specifically interact with the G-protein-coupled receptor 55 (GPR55) (Oka *et al*., 2007; Waldeck-Weiermair *et al*., 2008) that mediates a transient Ca^{2+} increase upon stimulation of endothelial cells with LPI (Henstridge *et al*., 2008; Lauckner *et al*., 2008; Waldeck-Weiermair *et al*., 2008). This observation indicates that vascular endothelial cells are targets of LPI and might further point to LPI as an important modulator of endothelial functions under physiological and pathological conditions. Notably, GPR55 activation resulted in the suppression of a reconstituted neuronal M-type K^+ current in GPR55 transfected HEK 293 cells (Lauckner *et al*., 2008), suggesting that GPR55 stimulation might have multiple consequences for the regulation of the membrane potential. It has been reported that several LPs such as lysophosphatidylcholin (LPC) exert significant effects on plasma membrane currents of endothelial as well as smooth muscle cells (Terasawa *et al*., 2002; Kuhlmannm *et al*., 2004) suggesting that LPs are capable of modulating vascular reactivity, predominantly by affecting ionic homeostasis in vascular cells. In vascular endothelial cells, the membrane potential is crucially important for various cell functions, including Ca^{2+} entry (Nilius and Droogmans, 2001), nitric oxide formation and the generation of endothelium-derived hyperpolarizing factor (EDHF; Chen and Suzuki, 1990; Graier *et al*., 1996), and free-radical production (McCarty, 1999). Moreover, changes in endothelial membrane potential *in situ* via myo-endothelial gap junctions influence the membrane potential of underlying smooth muscle cells (Beny and Pacicca, 1994) and, hence, have profound influence on vascular tone.

Because little is known about the effects of LPI as a possible vascular signalling mediator on endothelial membrane potential, this study was designed to investigate the effects of LPI on intracellular Ca^{2+} concentration, membrane potential, and to explore the underlying ion conductance in endothelial cells.

Methods

Cell culture

The human umbilical vein derived endothelial cell line, EA.hy926 (Edgell *et al*., 1983) at passage >45 was grown in DMEM containing 10% FCS and 1% HAT (5 mM hypoxanthine, 20 μM aminopterin, 0.8 mM thymidine) and cells were maintained in an incubator at 37°C in 5% CO_2 atmosphere. Cells were plated on 30 mm glass cover slips at least 2 days before use in experiments (Paltauf-Doburzynska *et al*., 2000).

Cell transfection and small interference RNA

Adherent cells of approximately 70–80% confluence were transiently transfected with 2–3 μg cDNA of the respective vectors using TransFast™ Transfection Reagent (Promega, Mannheim, Germany) according to the instruction manual and as previously described (Trenker *et al*., 2007). Cells were used approximately 36 h after transfection. For the knock-down of GPR55 small interference RNA (siRNA) (Sense sequence of GPR55 siRNA: 5′-GGUUCUUGGCCAUCCGUUAtt-3′, Qiagen, Cambridge, MA) was used. Cells were transfected with either the siRNAs or the AllStars® negative control siRNA (Cat. No. 1027281, Qiagen, Cambridge, MA) as described previously (Trenker *et al*., 2007; Waldeck-Weiermair *et al*., 2008). Efficiency of the siRNA used was positively tested in our previous work (Waldeck-Weiermair *et al*., 2008). For GPR55, overexpression cells were transiently transfected with a vector encoding GPR55 as previously described (Waldeck-Weiermair *et al*., 2008).

Ca^{2+} measurements

Cytosolic free-Ca^{2+} was measured using Fura-2/AM as previously described (Graier *et al*., 1996; Paltauf-Doburzynska *et al*., 2000). Briefly, cells were loaded with 2 μM fura-2/AM for 45 min at room temperature. Prior to experiments, cells were washed and equilibrated for a further 20 min. Subsequently, cells were illuminated on an inverted microscope (Eclipse 300 TE, Nikon, Vienna) alternatively at 340 and 380 nm (filters: 340HTI15 and 380HTI15; Omega Optical Brattleboro, VT, USA) and emitted light was collected at 510 nm (510WB40 emission; Omega Optical) using a cooled charge-coupled device camera (–30°C; Quantix KAF 1400G2, Roper Scientific, Acton, MA, USA). All Ca^{2+} measurements were performed with a 40× 1.3 N.A. oil-immersion objective (Plan Fluor, Nikon, Vienna) (Paltauf-Doburzynska *et al*., 1998; Frieden and Graier, 2000).

Electrophysiological recordings

Whole cell membrane currents and membrane potential were recorded using the perforated patch-clamp technique (Frieden and Graier, 2000; Bondarenko, 2004; Bondarenko and Sagach, 2006). The conventional whole cell technique was used only in those experiments in which cells were dialysed with Cs^+ -based solution. For membrane perforation, amphothericin B (300 μM) was included into the pipette solution. Single channel activity of large conductance, Ca^{2+}-activated, K^+ channels (BK_{Ca} channels; nomenclature follows Alexander *et al*., 2009) was recorded in the cell-attached mode (Malli *et al*., 2003). Membrane currents and potential were recorded using a List EPC7 amplifier (List, Germany). Borosilicate glass pipettes were pulled with a Narishige puller

(Narishige Co. Ltd, Tokyo, Japan), fire-polished and had a resistance of 4–6 MΩ. The signals obtained were low pass filtered at 1 kHz, and digitized with a sample rate of 10 kHz using a Digidata 1200A A/D converter (Axon Instruments, Foster City, CA, USA). Data collection and analysis were performed using Clampex and Clampfit software of pClamp (version 8.2, Axon Instruments). The holding potential in whole cell experiments was –40 mV. Voltage ramps of 1 s duration from –80 mV to +80 mV were delivered every 5 s.

Solutions
The standard external solution contained (in mM): 145 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, 2.4 $CaCl_2$ (extracellular buffer #1, EB#1). In Ca^{2+}-free solutions, $MgCl_2$ was increased to 2.2 mM and 1 mM EGTA was added. Patch pipettes were filled with a solution containing (in mM): 100 potassium aspartate (KAsp), 40 KCl, 10 HEPES, 2 $MgCl_2$, 0,2 EGTA (pipette solution #1, PS#1). For studying the Na^+-dependency of LPI-induced responses, bath solution contained: 150 NaCl, 1,2 $MgCl_2$, 10 glucose, 10 HEPES, 1 EGTA (extracellular buffer #2, EB#2) and after current development in response to LPI, Na^+ was omitted by substituting with choline. In these experiments a Cs^+-based pipette solution contained the following components (in mM): 100 $CsMeSO_3$, 40 CsCl, 10 HEPES, 2 $MgCl_2$, 10 EGTA (pipette solution #2, PS#2). For single channel recordings of BK_{Ca} channels in the cell-attached mode, the bath solution contained (in mM): 150 KCl, 10 HEPES, 10 glucose, 5 EGTA and 4931 mM $CaCl_2$ to set free Ca^{2+} concentration at 10 μM (calculated by the programme CaBuf, G. Droogmans, Leuven, Belgium; ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip) (extracellular buffer #3, EB#3) Pipette solution contained (mM): 100 KAsp, 40 KCl, 10 HEPES, 2 $MgCl_2$, 5 EGTA and 1.924 $CaCl_2$ to set free Ca^{2+} concentration to 100 nM (pipette solution #3, PS#3). Pipette solution in inside-out experiments for the activation of non-selective cation channels contained (in mM): 140 NaCl, 5 KCl, 2,4 $CaCl_2$, 1,2 $MgCl_2$, 10 HEPES and 10 D-glucose (pipette solution #4, PS#4). The respective bath solution contained (in mM): 140 CsCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA and free Ca^{2+} concentration was set to 100 nM by adding 1.924 $CaCl_2$ (extracellular buffer #4, EB#4). The pH of the pipette solutions was adjusted to 7.25 with either KOH or CsOH. The pH of the external solutions was adjusted to 7.40 with either NaOH or CsOH (for Na^+-free solution).

Intracellular Na^+ measurements
Cytosolic free Na^+ was measured using CoroNa™ Green (Meier *et al*., 2006) following the standard procedure (Ohta *et al*., 2008). Briefly, cells were loaded with 5 μM CoroNa™ Green/AM for 30 min at room temperature followed by an equilibration period of 20 min. After washing the cells three times, cells were illuminated on an inverted microscope at 433 nm (433DF15, Omega Optical) and emission was collected at 535 nm (535AF26, Omega Optical, Brattleboro, USA). Changes in cytosolic Na^+ were expressed as $\Delta F_{430}/F_0$.

Statistics
Analysis of variance was performed and statistical significance was verified using Scheffe's *post hoc F*-test. Level of significance was defined as $P < 0.05$.

Materials
Fura-2/AM and CoroNa™ Green/AM, gramicidin and cell culture chemicals were obtained from Invitrogen (Vienna, Austria). Fetal bovine serum was from PPA Laboratories (Linz, Austria). LPI, Dulbecco's modified Eagle's medium (DMEM) and all other chemicals were purchased from Sigma (Vienna, Austria).

Results

LPI elicits biphasic Ca^{2+} elevation, accompanied by diverse changes in membrane potential
In the presence of extracellular Ca^{2+}, cell stimulation with 5 μM LPI induced a transient rise in cytosolic free [Ca^{2+}], which returned to the basal level within 2–4 min even in the presence of 2 mM extracellular Ca^{2+} (Figure 1A). The comparison of LPI-induced Ca^{2+} signalling in the presence of extracellular Ca^{2+} with its effect in nominal Ca^{2+}-free solution (Figure 1B) indicated that LPI mainly mobilized Ca^{2+} from internal Ca^{2+} stores, whereas Ca^{2+} entry contributed only marginally to the cytsolic Ca^{2+} elevation in this early phase while the sustained Ca^{2+} rise reflected Ca^{2+} entry. The concentration-response analysis in respect of cytosolic Ca^{2+} elevation in response to LPI revealed the initial intracellular Ca^{2+} mobilization to be more sensitive than the sustained Ca^{2+} entry (Figure 1C).

The initial cytosolic Ca^{2+} elevation upon LPI in the presence of extracellular Ca^{2+} was accompanied by a transient hyperpolarization that reached maximal amplitude of 11.4 ± 1.7 mV ($n = 9$) within 100 s. Following the initial hyperpolarization, a slowly developing sustained depolarization of 20.1 ± 2.5 mV ($n = 9$) above the resting membrane potential occurred within 250–300 s (Figure 1D). The concentration-response analyses revealed similar sensitivities of the initial hyperpolarization and subsequent depolarization (Figure 1E) compared with the respective Ca^{2+} signals (Figure 1C).

Upon repetitive applications, the LPI-induced initial hyperpolarization was markedly reduced or absent while the sustained depolarization remained unchanged (Figure 1F). In agreement with these findings, LPI failed to initiate repetitively the respective outward current that accompanied membrane hyperpolarization upon the first stimulation while a sustained inward current always occurred upon any LPI stimulation (Figure 1G).

GPR55 is involved in the initial hyperpolarization but not the sustained depolarization in response to LPI
Because in the cell model used, GPR55 was found to be constitutively expressed and to account for a fast, transient Ca^{2+} elevation upon stimulation with LPI (Waldeck-Weiermair *et al*., 2008), the contribution of this receptor to LPI-induced Ca^{2+} signalling and electrical responses in endothelial cells was further explored. In agreement with these findings, the

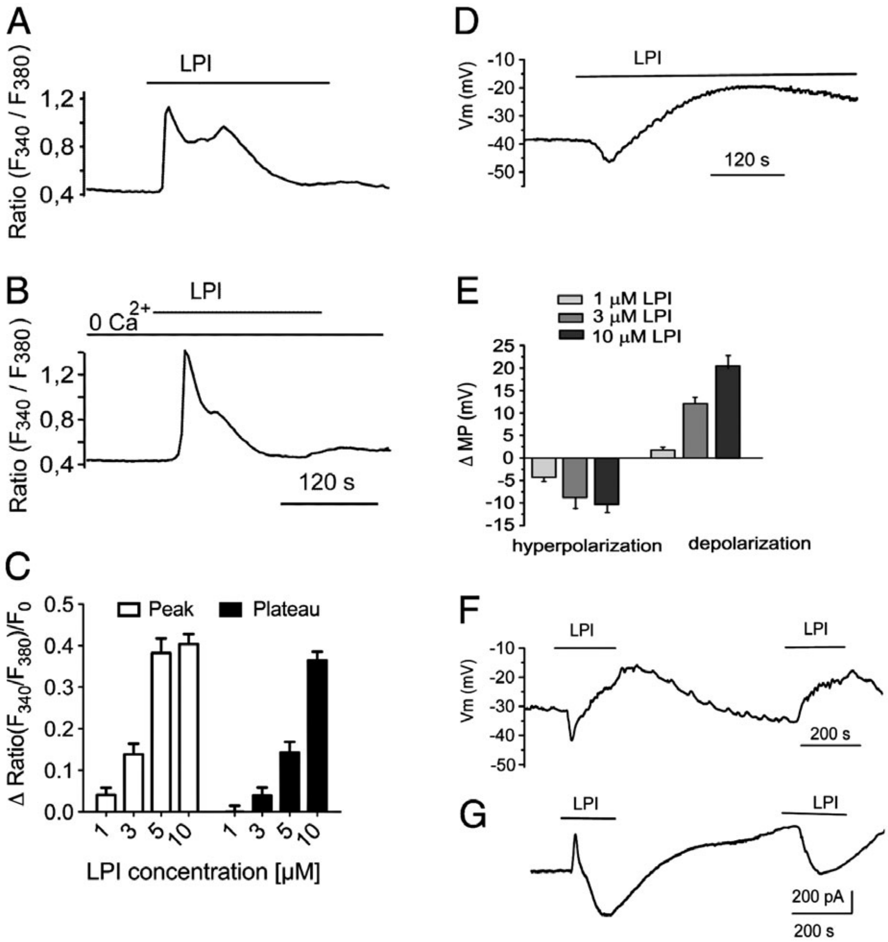

Figure 1 Effect of LPI on free intracellular Ca^{2+} and membrane potential of endothelial cells. Representative effect of 5 μM LPI on free intracellular Ca^{2+} in the presence of 2 mM extracellular Ca^{2+} (n = 32) (A) and in nominally Ca^{2+}-free solution (n = 27) (B). Concentration-response correlation of LPI on cytosolic Ca^{2+} concentration measured at the initial transient peak (Peak Phase) and the subsequent plateau phase (Plateau Phase) (1 μM, n = 9; 3 μM, n = 9; 5 μM, n = 15; 10 μM, n = 14) (C). Representative biphasic effect of LPI (5 μM) on membrane potential in the presence of extracellular Ca^{2+} (n = 9) (D). Concentration-response correlation of LPI in terms of initial membrane hyperpolarization and subsequent depolarization (1 μM, n = 17; 3 μM, n = 7; 10 μM, n = 7) (E). Representative changes in endothelial membrane potential evoked by repetitive stimulations with 5 μM LPI (n = 5) (F). Representative membrane currents evoked by repetitive stimulations by LPI (5 μM) at −40 mV holding potential (n = 3) (G).

GPR55 blocker rimonabant (1 μM) (Lauckner *et al.*, 2008; Waldeck-Weiermair *et al.*, 2008) diminished the LPI-initiated immediate Ca^{2+} transient (Figure 2A). Moreover, rimonabant (1 μM) prevented the transient membrane hyperpolarization (Figure 2B,C) and outward current in response to 5 μM LPI (Figure 2D). In contrast, the sustained membrane depolarization (Figure 2B,C) and inward current (Figure 2B) in response to LPI were not affected by the GPR55 blocker.

However, there is some controversy over to the pharmacological potential of putative inhibitors/activators of GPR55 (see Ryberg *et al.*, 2007; Henstridge *et al.*, 2008; Waldeck-Weiermair *et al.*, 2008; Kapur *et al.*, 2009; Ross, 2009; Yin *et al.*, 2009). So, the involvement of GPR55 was further investigated by altering expression of GPR55. In line with these findings and our previous work using higher LPI concentrations (Waldeck-Weiermair *et al.*, 2008), intracellular Ca^{2+} release triggered by LPI (5 μM) was strongly enhanced in cells that over-expressed GPR55 (Figure 3A) while this response was strongly attenuated in cells treated with siRNA against GPR55 (Figure 3B). Moreover, the initial membrane hyperpolarization in response to LPI was reduced in siRNA treated cells and augmented when GPR55 was over-expressed (Figure 3C). In contrast, the subsequent membrane depolarization remained unaffected [Control: 10.50 ± 2.10 mV (n = 4); GPR55 over-expressing cells: 12.16 ± 2.20 mv (n = 6)].

To further assess the mechanistic differences between LPI-triggered initial hyperpolarization and the long-lasting membrane depolarization, the effect of the phospholipase C inhibitor U73122 (Figure 4A) and of the G protein inhibitor GDPβS (Figure 4B) was tested in conventional patch clamp experiments. U73122 as well as GDPβS prevented LPI- (3 μM) induced membrane hyperpolarization while the depolarizing effect of LPI remained unaffected in the presence of GDPβS. Moreover, in inside-out experiments LPI in the bath activated a non-selective ion current (Figure 4C), thus, indicating that LPI, besides its stimulatory activity at GPR55, might act independently from a receptor directly on plasma membrane ion channels.

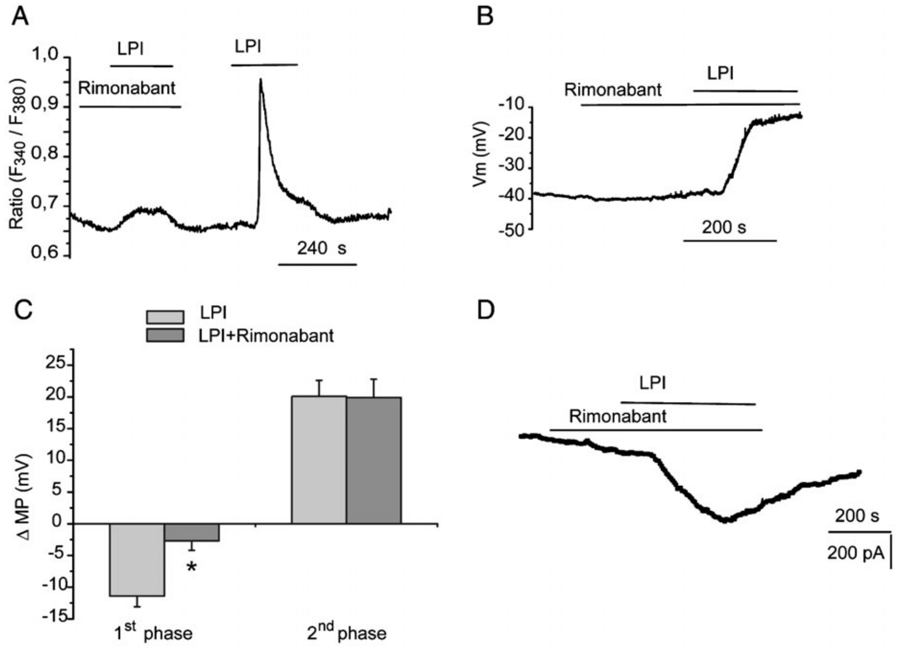

Figure 2 The GPR55 blocker rimonabant prevented only the initial Ca^{2+} transients and membrane hyperpolarization evoked by LPI. Cells were pre-treated with 1 μM rimonabant as shown. This GPR55 blocker abolished the transient elevation of the cytosolic-free Ca^{2+} concentration in response to 5 μM LPI (A). Typical membrane potential recording showing the inhibitory effect of rimonabant (1 μM) of a LPI (5 μM)-evoked hyperpolarization (B). Statistical evaluation of LPI (5 μM)-induced biphasic changes in endothelial cell membrane potential under control conditions (LPI, $n = 9$) and in the presence of 1 μM rimonabant ($n = 14$) (C). Rimonabant (1 μM) prevented the development of the initial outward current (1st phase) but failed to prevent the sustained inward current (2nd phase) in response to 5 μM LPI ($n = 3$) (D). *$P < 0.005$ versus control.

These data indicate that the initial and transient ionic responses to LPI essentially depend on GPR55, while the subsequent, slowly developing, sustained electrical responses appear to be independent of this orphan receptor and mediated via direct effects on non-selective cation channels in endothelial cells.

LPI-induced changes in membrane potential are due to different currents

To describe the transmembrane currents that underlie the LPI-induced changes in membrane potential, experiments in the voltage clamp mode were performed. When membrane voltage was kept at –40 mV, which is close to the resting membrane potential (–40.9 ± 2.2 mV; $n = 23$) of this cell type, 5 μM LPI induced a biphasic response that showed a transient outward current (*Hyperpolarization phase*) followed by a sustained inward current (*Depolarization phase*) (EB#1, PS#1) (Figures 1G and 5A).

Hyperpolarization phase. Voltage ramps between –80 to +80 mV during the hyperpolarization (outward current) phase (Figure 5A) revealed a current potential curve with the common characteristics of an agonist-activated current, mainly achieved by BK_{Ca} channels in whole cell recordings of these endothelial cells (Figure 5B) (Frieden and Graier, 2000). In agreement with this assumption, LPI induced rapid activation of large conductance channels in the cell-attached configuration (Figure 5C). In the whole-cell configuration, the outward current did not appear when pipettes were filled with Ca^{2+}-free Cs^{+}-containing solution (PS#2) in order to avoid K_{Ca} currents (data not shown). Moreover, membrane hyperpolarization to LPI was sensitive to charybdotoxin (Figure 5D). Charybdotoxin (100 nM) produced a gradual endothelial depolarization (Figure 5D) that points to the contribution of charybdotoxin-sensitive K_{Ca} channels to the resting membrane potential (Ledoux *et al*., 2008). Altogether, these results indicate that the LPI-induced GPR55-dependent hyperpolarization is due to an activation of large conductance, Ca^{2+}-dependent, charybdotoxin-sensitive, K^{+} channels.

Depolarization phase. Next, the sustained inward current that accounts for the slowly developing membrane depolarization in endothelial cells in response to LPI was investigated. Using voltage ramps between –80 to +80 mV in the inward current phase (Figure 6A) revealed a linear current-potential curve (Figure 6B). We intended to characterize the nature of the current(s) that was/were responsible for the LPI-induced membrane depolarization in endothelial cells. First, the involvement of Cl^{-} channels in LPI-induced depolarization was tested using the Cl^{-} channel blocker 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). DIDS (100 μM and 1 mM) failed to prevent endothelial depolarization (Figure 6C) evoked by 5 μM LPI, thus, suggesting that Cl^{-} channels are unlikely to be involved in the LPI-induced electrical responses in endothelial cells.

To evaluate the role of Ca^{2+} entry in the LPI-induced electrical responses, LPI was added either in nominal Ca^{2+}-free or Ni^{2+}-containing solution. Ca^{2+} withdrawal evoked a gradual depolarization with the amplitude of 11.4 ± 2.1 mV ($n = 6$). In

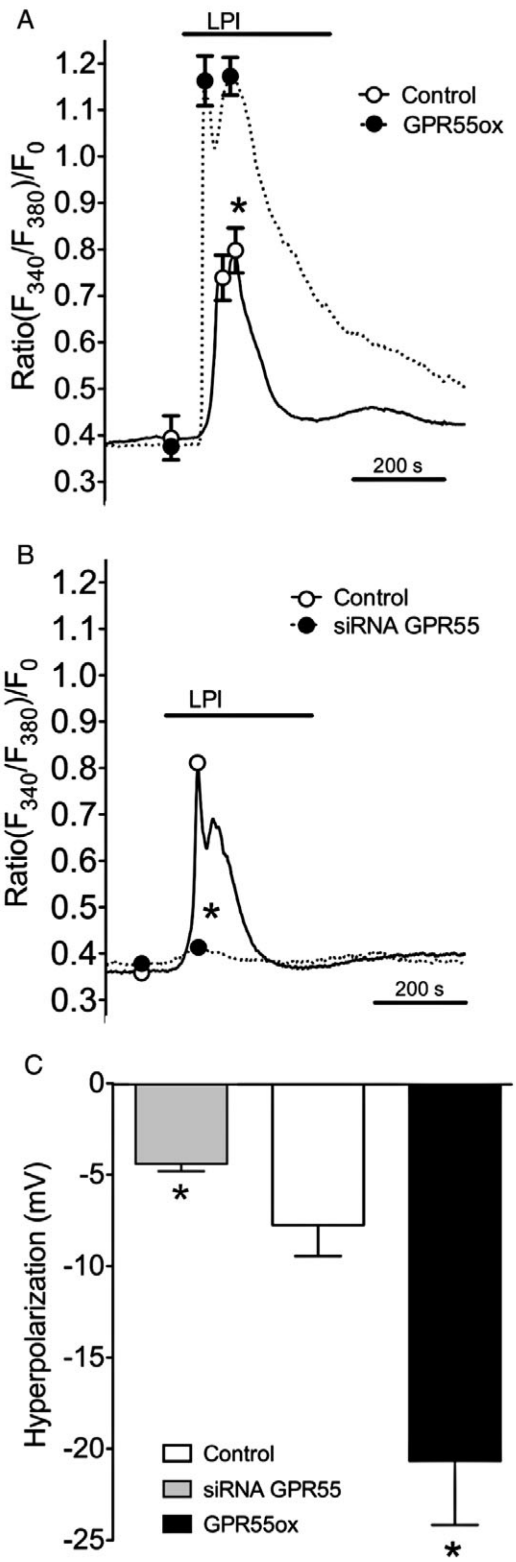

Figure 3 The effects of LPI on initial Ca^{2+} signalling and membrane hyperpolarization essentially depend on GPR55 expression. Intracellular Ca^{2+} signalling in response to 5 μM LPI was recorded in cells transiently transfected with the respective controls (same vector encoding nuclear GFP) or a GPR55 encoding vector (Control: $n = 17$; GPR55ox: $n = 24$) (A). In addition, cells were transfected with siRNA control (AllStar®) or siRNA against GPR55 (Control: $n = 12$; siRNA GPR55: $n = 19$) (B). In conventional whole cell configuration, the LPI- (5 μM) induced hyperpolarization was measured in cells transfected either siRNA control ($n = 4$), siRNA against GPR55 ($n = 5$) or a vector encoding GPR55 ($n = 6$) (C). *$P < 0.005$ versus control.

the absence of extracellular Ca^{2+}, LPI produced a more pronounced initial hyperpolarization with the mean amplitude of −15.4 ± 2.7 mV ($n = 5$) (Figure 7A). However, the subsequent depolarization in Ca^{2+}-free solution was reduced by about 42 ± 4% ($n = 5$) compared with the depolarizing effect of LPI in Ca^{2+} containing buffer (Figure 7A,F). Ca^{2+} re-addition in the continued presence of LPI produced a hyperpolarization followed by a depolarization above the level attained in the presence of LPI in Ca^{2+}-free solution (Figure 7B,F). Similar results were obtained if Ca^{2+} entry was prevented by 2 mM Ni^{2+} (Figure 7C,F). Addition of Ni^{2+} caused a gradual depolarization pointing to a basal Ca^{2+} entry as a determinant of the resting membrane potential in this cell type. Subsequent addition of 5 μM LPI evoked a slightly increased amplitude of the transient hyperpolarization (18.7 ± 2.4 mV, $n = 7$) followed by a sustained depolarization that was significantly reduced by 51 ± 4% compared with the corresponding experiments that were performed in the absence of Ni^{2+} (Figure 7C,F). In line with these findings, the LPI-induced inward current (repetitive stimulation) was reduced upon addition of 2 mM Ni^{2+} into the bath by 50 ± 6% (Figure 7D). Next, we examined the sensitivity of the depolarization phase to La^{3+}, a known blocker of store-operated and non-selective cation channels (Nilius and Droogmans, 2001). In these experiments, an addition of 100 μM La^{3+} during the sustained depolarization upon a repetitive LPI-administration (5 μM) reduced membrane depolarization by 53 ± 7% (Figure 7E,F).

These results indicate that the lack of Ca^{2+} entry slightly facilitates LPI-induced hyperpolarization and reduces the subsequent sustained depolarization phase, indicating that Ca^{2+} entry partially (approximately 50%) accounts for the depolarizing effect of LPI. Together with the current-potential relationship of the current that is associated with the sustained depolarization phase upon LPI stimulation (Figure 6B), these results point to the involvement of Ca^{2+} permeable, non-selective channels, activated by LPI action and contributing to LPI-induced endothelial cell depolarization.

In order to study whether or not Na^+ entry via such non-selective cation channels also contributes to the LPI-induced depolarization, experiments in the absence of extracellular Na^+ (Na^+ substituted for choline) and nominal Ca^{2+}-free buffer were performed. Under such conditions, LPI-induced sustained depolarization was reduced by 94 ± 3% compared with the respective control (Figure 8A). In voltage-clamp experiments (PS#2, holding potential −40 mV, voltage ramps from −80 to +80 mV), LPI-induced inward current was abolished upon substitution of extracellular Na^+ with equimolar choline (Figure 8B). These data suggest that Ca^{2+}- and Na^+-permeable non-selective cation channels account for the slowly developing but sustained depolarization upon stimulation of LPI.

In line with the findings above, LPI resulted in a slow accumulation of intracellular Na^+ (Figure 8C), This effect was

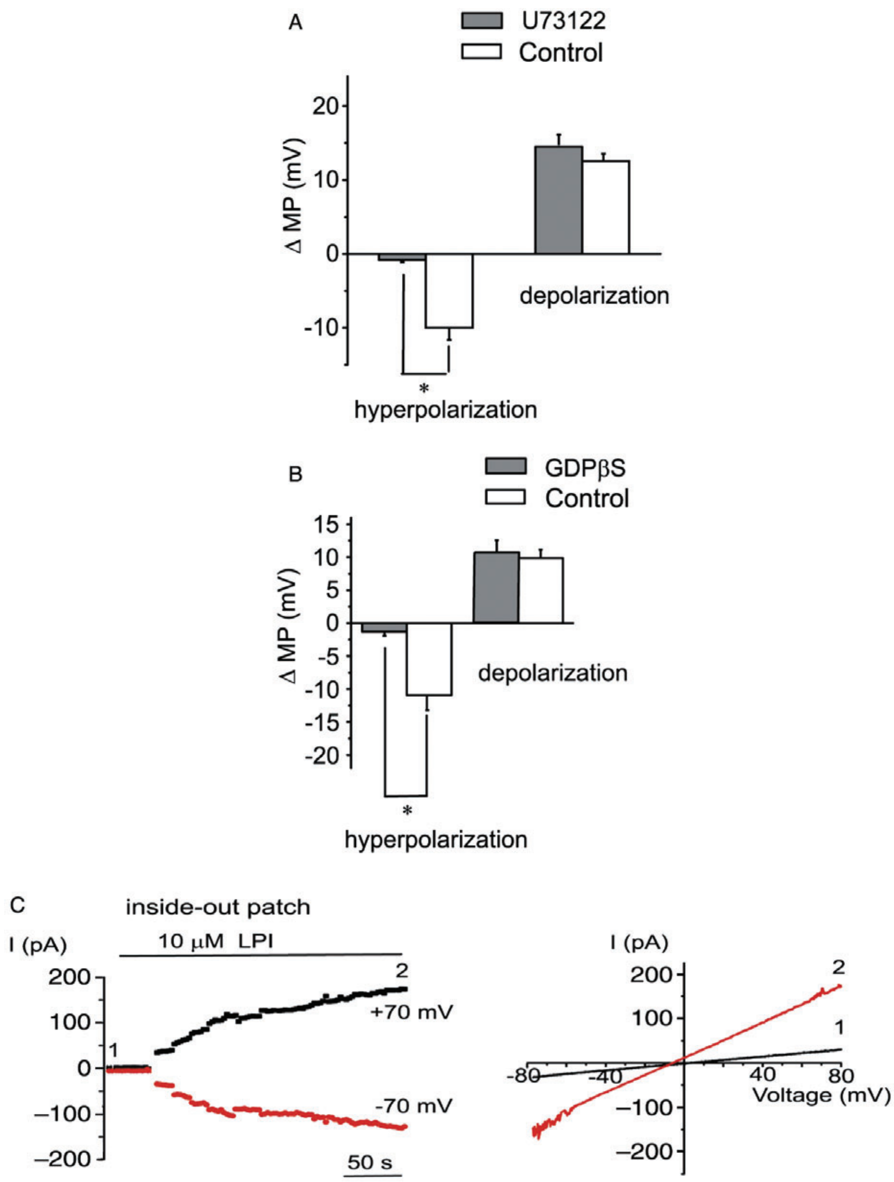

Figure 4 LPI-induced hyperpolarization but not depolarization is sensitive to inhibition of phospholipase C and to GDPβS. In conventional whole cell configuration, the effect of the inhibitor of phospholipase C U73122 (10 μM) (Control: n = 5; U73122: n = 6) (A) or 1 mM GDPβS in the pipette (Control: n = 7; GDPβS: n = 12) (B) on LPI- (3 μM) evoked initial hyperpolarization and subsequent depolarization was evaluated. *$P < 0.005$ versus control. The effect of 10 μM LPI in the bath in the inside-out configuration (PS#4) in the presence of 100 nM free Ca^{2+} (EB#4) on plasma membrane currents at +70 and −70 mV membrane potentials (*left*) and the respective voltage-current relationship before (1) and after (2) LPI application (*right*) (n = 6) were recorded (C).

independent from GPR55 as indicated by the lack of an effect of GPR55 knock-down on LPI-triggered cytosolic Na^+ elevation (Figure 8C). The concentration dependency that indicated a great difference in the effects initiated by 5 versus 10 μM LPI (Figure 8D, *left*) was similar to that obtained for Ca^{2+} entry/plateau phase (Figure 1C) and membrane depolarization (Figure 1E). In the absence of extracellular Na^+, LPI failed to increase cytosolic Na^+ levels (Figure 8D, *right*).

LPI-evoked sustained membrane depolarization is partially due to inhibition of Na/K-ATPase

Because in our experiments above, LPI-induced sustained membrane depolarization dependent on Ca^{2+}/Na^+ inward currents (Figures 6–8), further experiments were performed in order to assess the possible involvement of the Na/K-ATPase in LPI-induced depolarization. First, 5 μM LPI prevented, in a reversible manner, membrane hyperpolarization upon re-adddition of extracellular K^+ (Figure 9A), a standard protocol that reveals Na/K-ATPase-dependent repolarization upon the re-addition of K^+ (Bondarenko and Sagach, 2006). Furthermore, preincubation of endothelial cells with 250 μM ouabain, which yielded strong membrane depolarization, reduced LPI (5 μM)-induced sustained depolarization from 20.1 ± 2.5–8.5 ± 1.5 mV (n = 9) (Figure 9B,C). The subsequent inhibition of the non-selective cation channels by 100 μM La^{3+} in the presence of ouabain completely prevented LPI-induced, sustained membrane depolarization (Figure 9B,C).

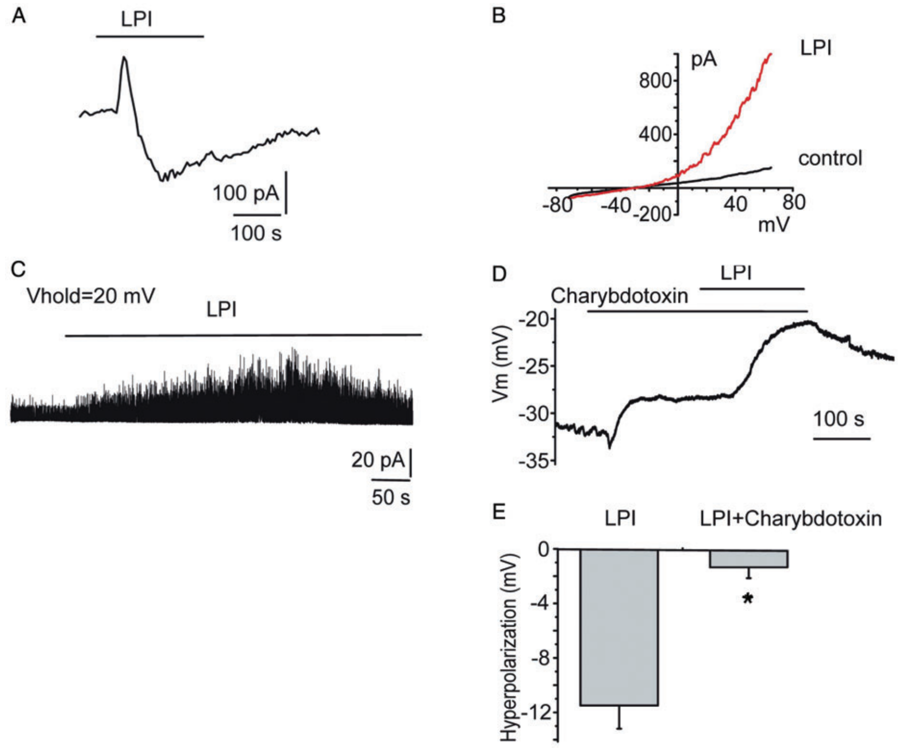

Figure 5 The initial transient response to LPI is due to a stimulation of outwardly rectifying, Ca^{2+}-dependent, charybdotoxin-sensitive, K^+ channels. Representative biphasic changes in membrane currents evoked by 5 μM LPI at a holding portential of –40 mV (*n* = 14) (A). Voltage ramps of the outward rectifying membrane currents under resting conditions (control, *n* = 5) and upon cell stimulation with 5 μM (LPI (*n* = 8) (B). Representative time course of the effect of LPI on single BK_{Ca} channels in the cell-attached mode at a holding potential (Vhold) of 20 mV (*n* = 7) (C). Representative membrane potential recording showing a lack of hyperpolarization in response to 5 μM LPI in the presence of 100 nM charybdotoxin (*n* = 5) (D). Statistical evaluation of LPI (5 μM)-induced membrane hyperpolarization in endothelial cells under control conditions (LPI, *n* = 4) and in the presence of 100 nM charybdotoxin (100 nM, *n* = 5) (E). $^*P < 0.005$ versus control.

Discussion

The present study demonstrated that the endogenous agonist of GPR55, LPI induced a variety of electrical responses in endothelial cells. The immediate and rather transient Ca^{2+} elevation, and its associated K^+ current and membrane hyperpolarization to LPI were mediated via GPR55. However, the slowly developing and sustained inward current that resulted in intracellular Na^+ loading and membrane depolarization was independent of GPR55 and was due to inhibition of Na/K-ATPase and direct activation of non-selective cation channels. Thus, LPI is a potent signalling molecule affecting endothelial cells by modulating multiple electrical responses. Because, these effects are only partially linked to GPR55, the physiological meaning of LPI as an intercellular messenger within the vascular wall exceeds that of just an activator of GPR55. Accordingly, in the ongoing evaluation of the physiological role of GPR55 and its endogenous agonists, these biphasic responses need to be considered.

LPI recently received much attention following several concordant reports that identified LPI but not other lysophospholipids, such like lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine or lysophosphatidic acid, as endogenous activators of GPR55 (Oka *et al*., 2007; 2008; Henstridge *et al*., 2008; Waldeck-Weiermair *et al*., 2008). Because the physiological concentrations of LPI were found to be 1–15 μM (Xiao *et al*., 2001), the LPI concentrations we used here are close to those found *in vivo*. There is an ongoing discussion whether or not GPR55 serves as receptor for endocannabinoids, such as anandamide and 2-arachidonyl glycerol, though it obviously shares some similar pharmacology with classical cannabinoid receptors (Ross, 2009), The two putative endogenous GPR55 agonists, LPI and anandamide, share certain biological activities such as stimulation of intracellular Ca^{2+} signalling (Howlett and Mukhopadhyay, 2000; Oka *et al*., 2007; Henstridge *et al*., 2008; Lauckner *et al*., 2008; Waldeck-Weiermair *et al*., 2008) and the initiation of mitogenic signals (Falasca and Corda, 1994; Corda *et al*., 2002; Waldeck-Weiermair *et al*., 2008). Regardless of their proliferative potential, anandamide and LPI predominantly exhibit inhibition of endothelial cell migration, tumour growth and angiogenesis (Murugesan and Fox, 1996; Flygare and Sander, 2008).

Endothelial cells express functional GPR55 (Waldeck-Weiermair *et al*., 2008) and produce its endogenous agonist, LPI (Hong *et al*., 1985; Kaya *et al*., 1989), making it more likely that LPI could be an intra-vascular messenger. To understand the physiological role of LPI, an in-depth understanding of the signalling cascades initiated by LPI in endothelial cells is essential. In this study, LPI was found to exhibit several autonomous effects in endothelial cells of which the immediate, rather transient signalling depended exclusively on

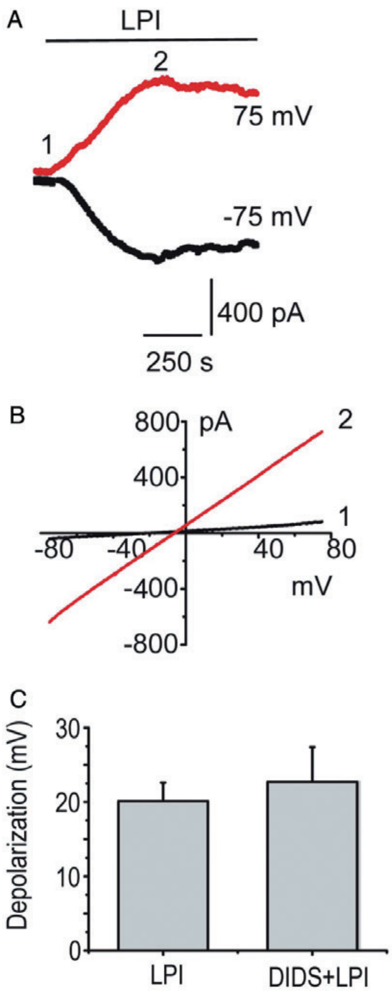

Figure 6 LPI-evoked membrane currents that account for membrane depolarization. Representative tracings of membrane currents induced by 5 μM LPI measured at holding potentials of –75 and +75 mV (n = 7). In the whole-cell mode, 1 s voltage ramps from –80 mV to +80 mV were applied every 5 s from a holding potential of –40 mV (A). Current-voltage relationship constructed from current responses to voltage ramps before and after application of 5 μM LPI at points specified in A (B). Statistical evaluation of the effects of 5 μM LPI on endothelial cell membrane potential under control conditions (n = 7) and in the presence of 100 μM DIDS (n = 3) (C).

GPR55. This conclusion is based on our findings that the antagonist of GPR55, rimonabant (Lauckner *et al*., 2008; Waldeck-Weiermair *et al*., 2008) and siRNA-mediated GPR55 knock-down prevented the rapid intracellular Ca^{2+} release in response to LPI, confirming our earlier results that LPI triggered intracellular Ca^{2+} mobilization via GPR55 (Waldeck-Weiermair *et al*., 2008). Moreover, our findings that the inhibition/knock-down of GPR55 prevented LPI-induced transient membrane hyperpolarization and outward current while GPR55 over-expression strongly enhanced LPI-induced hyperpolarization, further supports our assumption that the initial GPR55-mediated Ca^{2+} elevation/release is accompanied by activation of plasma membrane ion channels, that were characterized as intermediate and large conductance Ca^{2+}-activated K^+ channels, the main carrier for Ca^{2+}-induced membrane hyperpolarization in this cell type (Frieden and Graier, 2000; Malli *et al*., 2003). In contrast to other G protein-coupled receptor agonists in this cell type (Malli *et al*., 2007), LPI initiated a transient Ca^{2+} elevation even in the presence of extracellular Ca^{2+}, which points to a limited activity of store-operated Ca^{2+} entry (SOCE). Nevertheless, LPI also induced inward currents that were not linked to increased cytosolic Ca^{2+}. These findings were in apparent contradiction to reports describing LPI as direct activator of SOCE in smooth muscle cells (Smani *et al*., 2003; 2007; Singaravelu *et al*., 2006) and the hypothesis that LPI represents a Ca^{2+}-independent, phospholipase A_2-derived, mediator for activation of SOCE (Smani *et al*., 2004). These differences might be due to the expression of a wide variety of Ca^{2+}-permeable ion channels in different cell types. Moreover, because LPI-triggered sustained membrane depolarization and inward current were not affected by inhibition of GPR55 by rimonabant or the molecular manipulation of GPR55 expression, LPI obviously exhibited its sustained effects independently of this orphan receptor. This assumption was further supported by our findings that LPI-induced slow cellular Na^+ loading was independent of the expression level of GPR55.

Accordingly, the LPI-induced biphasic responses of the membrane potential differs from the reported monophasic hyperpolarization elicited by LPC in endothelial cells (Erdogan *et al*., 2007) and coronary artery smooth muscle cells (Terasawa *et al*., 2002), which respond by a slowly developing depolarization due to activation of a non-selective cation current. However, the biphasic effect of LPI on electrical responses reported here resembles the effect of LPC on renal arterial smooth muscle cells (Jabr *et al*., 2000).

To investigate the nature of the GPR55-independent inward current, blockers of several endothelial transporters and channels were tested. As LPI-evoked depolarization was not affected by the Cl^- channel blocker DIDS, the involvement of Cl^- channels (Nilius and Droogmans, 2001) in the membrane depolarization to LPI is unlikely.

In contrast, depletion of Ca^{2+} in the bath solution, which produced gradual depolarization possibly due to inhibition of basal Ca^{2+} entry and a subsequent reduction of BK_{Ca} channel activity, strongly reduced LPI-induced depolarization. Moreover, the Ca^{2+} entry blocker Ni^{2+} produced a gradual depolarization and diminished LPI-induced depolarization, thus, indicating that LPI-induced sustained depolarization requires Ca^{2+} entry. We also used La^{3+}, which selectively inhibits SOCE and Ca^{2+} permeable non-selective cationic channels (Nilius and Droogmans, 2001; Terasawa *et al*., 2002) at low concentrations. Our findings that La^{3+} attenuated the LPI-induced depolarization further support our assumption of the importance of Ca^{2+} entry for LPI-induced depolarization and point to a GPR55-independent activation of Ca^{2+} permeable non-selective cationic channel(s) by LPI.

Our experiments further showed that LPI increased also the Na^+ permeability of endothelial cells. After cell dialysis with Cs^+- containing solution, LPI evoked a linear current that reversed at 0 mV, a property of non-selective cationic current (Jabr *et al*., 2000; Terasawa *et al*., 2002). Na^+ substitution with choline inhibited the current, indicating a significant Na^+ permeability stimulated by LPI, which contributed to membrane depolarization. Furthermore, a direct slow and GPR55-independent Na^+ loading was observed in response to LPI,

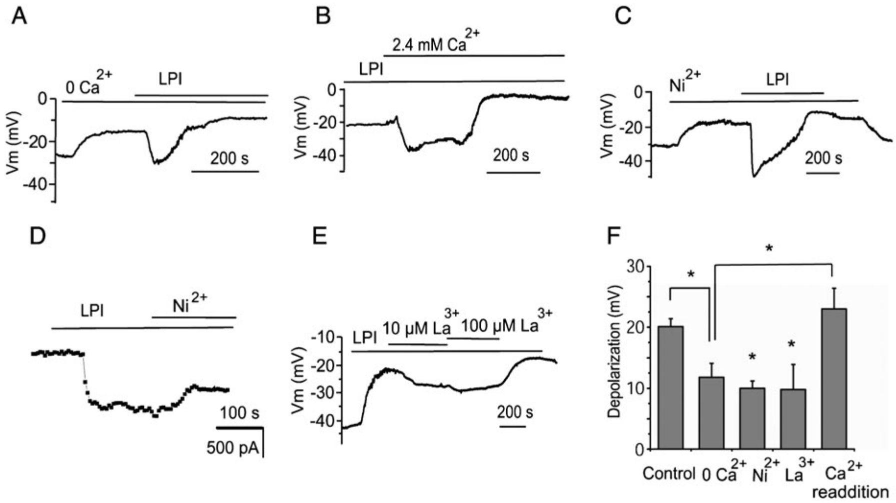

Figure 7 Dependency of the LPI-evoked electrical responses on extracellular Ca^{2+}. Original tracing of membrane potential fluctuations in response to 5 μM LPI in the absence of extracellular Ca^{2+} ($n = 16$) (A). Representative effect of re-adding Ca^{2+} in the continued presence of 5 μM LPI on membrane potential ($n = 3$) (B). Original tracing of the effect of 5 μM LPI on endothelial membrane potential in the presence of 2 mM Ni^{2+} ($n = 7$) (C). Effect of 2 mM Ni^{2+} on the LPI-induced inward current measured at –75mV ($n = 10$) (D). Effect of 100 μM La^{3+} on LPI-evoked depolarization ($n = 4$) (E). Statistical evaluation of the effects of different ionic conditions on the depolarization evoked by 5 μM LPI in endothelial cells: Control ($n = 14$), in the absence of extracellular Ca^{2+} (0 Ca^{2+}, $n = 5$), in the presence of 2 mM Ni^{2+} (Ni^{2+}, $n = 10$) or 100 μM La^{3+} (La^{3+}, $n = 4$), and on Ca^{2+} re-addition (Ca^{2+}, $n = 3$) (F). *$P < 0.05$ versus control.

indicating that LPI, in a GPR55-independent manner, induces a Ca^{2+}/Na^{+}-permeable non-selective cation channel in endothelial cells that favours Na^{+} to enter the cell and, thus, to initiate depolarization in the absence of an increase in cytosolic free Ca^{2+} concentration.

Removal of extracellular Ca^{2+} or the application of inhibitors of (non-selective) Ca^{2+}-permeable cation channels (Ni^{2+}, La^{3+}) only prevented about 50% of the depolarization to LPI. These data point to an additional GPR55-independent effect of LPI besides the activation of a non-selective cation channel. Because Ni^{2+} is also an inhibitor of the plasma membrane Na^{+}/Ca^{2+} exchanger (Iwamoto *et al*., 1999), this carrier can be excluded. Notably, LPI failed to trigger membrane depolarization in the absence of Na^{+}, indicating that the remaining membrane depolarization upon LPI relies on Na^{+}. Because other LPs including LPC and sphingosine exhibit inhibitory properties on the Na/K-ATPase (Oishi *et al*., 1990), we also evaluated the Na/K-ATPase as a putative target of LPI. Implementation of K^{+} re-addition protocols (Bondarenko and Sagach, 2006) demonstrated that, in the presence of LPI, a K^{+} -induced hyperpolarization was strongly reduced, indicating that LPI inhibited the Na/K-ATPase. In line with these findings, LPI-induced Ni^{2+}/La^{3+}-insensitive depolarization was prevented by ouabain, an inhibitor of the Na/K-ATPase. Moreover, only the combination of Ni^{2+}/La^{3+} and ouabain completely prevented the sustained deplolarization induced by LPI.

Although the function of GPR55 as a common binding site for LPI and anandamide was only demonstrated in regard to inositol 1,4,5-trisphosphate (IP_3)- dependent intracellular Ca^{2+} signalling (Oka *et al*., 2007; Lauckner *et al*., 2008; Waldeck-Weiermair *et al*., 2008), it is tempting to speculate that several other physiological effects of both compounds are due to their common action on GPR55. However, these compounds considerably differ in terms of their GPR55-independent potential to affect distinct ion channels and carriers and their role in the control of blood vessel tone. In particular, LPI was described as a phospholipase A_2-derived messenger for the activation of SOCE (Smani *et al*., 2003; 2007; Singaravelu *et al*., 2006), and to activate non-selective cation channels and to prevent Na/K-ATPase activity (this study). Anandamide was described to activate arachidonic acid-regulated Ca^{2+}-selective channels (Shuttleworth *et al*., 2004) and the transient receptor potential cation channel, TRPV1 (Zygmunt *et al*., 1999), and to trigger formation of 5,6-epoxyeicosatrienoic acid as a messenger for Ca^{2+}-influx (Graier *et al*., 1995; Hoebel *et al*., 1997). Thus, the differences in the physiological effects of these two putative endogenous GPR55 agonists are likely to be due to their different repertoire of GPR55-independent effects. Our present data on LPI-induced depolarization might explain the discrepancy between anandamide and LPI in their potential to control endothelium-dependent blood vessel tone: anandamide may act as an EDHF *per se* or be involved in its formation/release (see Randall and Kendall, 1998), while LPI inhibits acetylcholine-induced, endothelium-dependent, hyperpolarization of rat mesenteric arteries (Fukao *et al*., 1995).

In view of the reported more than 10-fold elevation of LPI in cancer-derived ascites fluid (Xiao *et al*., 2001), our data presented here deserve further attention. In particular, our findings that the GPR55-dependent effects of LPI tend to be more sensitive, compared with the GPR55-independent LPI effects, suggest a considerable shift in the biological activity of LPI, under certain conditions. However, this important aspect requires more extensive evaluation and exceeds the scope of the present work.

In conclusion, our results indicate that LPI exhibits GPR55-dependent and GPR55-independent effects in endothelial

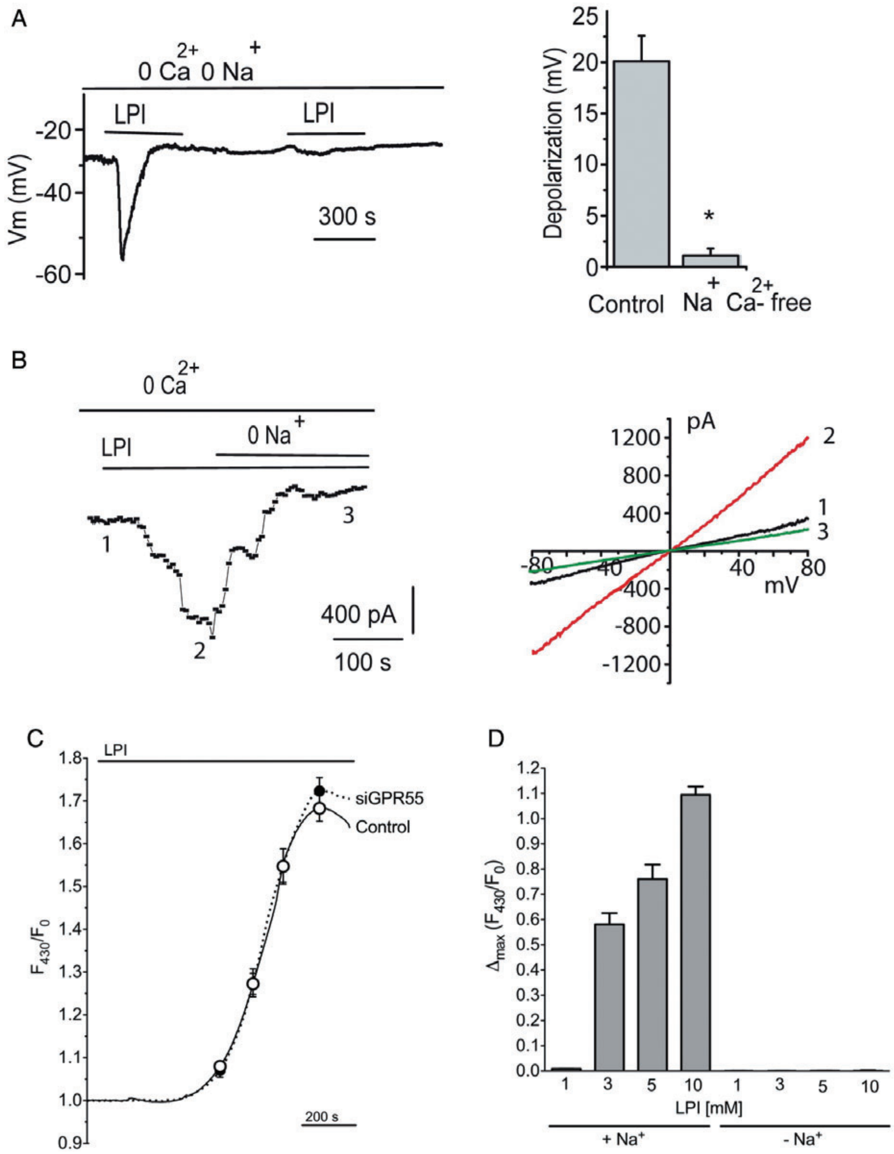

Figure 8 The LPI-evoked electrical responses depend on extracellular Na^+ and LPI-triggered, slow cytosolic Na^+ accumulation. Representative membrane potential recording showing LPI-evoked depolarization ($n = 6$) in the absence of Na^+ and Ca^{2+} in the bath (*left panel*) and the corresponding statistical evaluation of LPI (5 μM)-evoked depolarization under control conditions (Control) and in the absence of Na^+ and Ca^{2+} (*right panel*) (A). Representative recording of the inhibitory effect of Na^+ withdrawal on LPI-evoked inward current (*left panel*) measured at a holding potential of –40 mV ($n = 3$). Endothelial cells were dialyzed with a Cs^+- containing pipette solution and superfused with a Ca^{2+}- and K^+-free solution. Corresponding current-voltage relationship obtained from current responses to voltage ramps before and after the application of 5 μM LPI at time points indicated (*left panel*) (B). The effect of 5 μM LPI on intracellular Na^+ was recorded in CoroNa™ Green-loaded cells that were transiently transfected with either siRNA control (Control: $n = 17$) or siRNA against GPR55 ($n = 10$) (C). Concentration-response correlation of LPI on the accumulation of cytosolic Na^+ in a conventional buffer (EB#1) (*left*; $n = 15$–30) or in the absence of extracellular Na^+ (*right*; $n = 8$–15) (D). *$P < 0.005$ versus control.

cells. Because endothelial functions critically depend on Ca^{2+} entry and membrane potential fluctuations, modulation of these parameters by LPI points to a unique messenger function of LPI in the vasculature.

Acknowledgements

The authors thank Mrs Anna Schreilechner, BS, for her excellent technical assistance, Dr C.J.S. Edgell (University of North Carolina, Chapel Hill, NC, UCA) for the EA.hy926 cells and Dr M. Frieden (University of Geneva, Geneva, Switzerland) for critically reviewing this manuscript. This work was supported by the Austrian Funds (F3010-B05, P20181-B05 and P21857-B18).

Shamim Naghdi is funded by the FWF P20181-B05 within the PhD programme *Molecular Medicine* of the Medical University of Graz.

Conflicts of interest

None to declare.

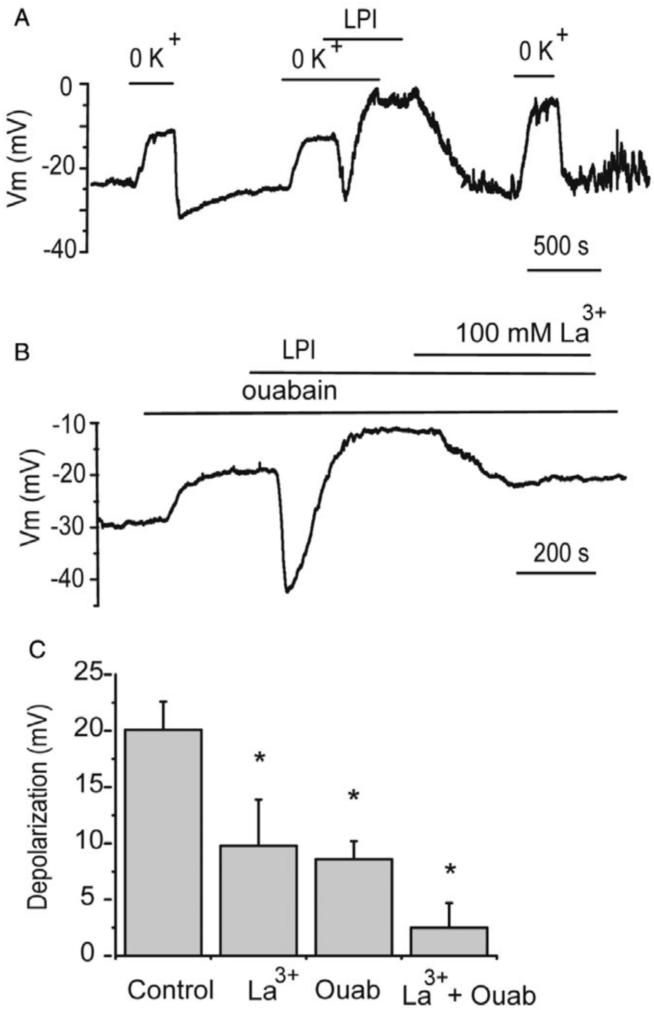

Figure 9 LPI-evoked sustained depolarization is partially due to inhibition of the Na^+/K^+-ATPase. Representative tracings of the endothelial cell membrane potential showing the inhibitory effect of 5 μM LPI on the hyperpolarization induced by re-adding K^+ to K^+-free solution (A) and the inhibitory effect of 250 μM ouabain on LPI-induced depolarization (B). Statistical evaluation of results from panels (A) and (B), showing the mean of the maximal membrane depolarization in response to 5 μM LPI in the presence of 100 μM La^{3+} ($n = 4$), 250 μM ouabain ($n = 9$), or 100 μM La^{3+} and 250 μM ouabain ($n = 3$) (C). *$P < 0.05$ versus control.

References

Alexander SPH, Mathie A, Peters JA (2009). Guide to receptors and channels (GRAC), 4th edn. *Br J Pharmacol* **158** (Suppl. 1): S1–S254.

Beny JL, Pacicca C (1994). Bidirectional electrical communication between smooth muscle and endothelial cells in the pig coronary artery. *Am J Physiol* **266** (4 Pt 2): H1465–H1472.

Bondarenko A (2004). Sodium-calcium exchanger contributes to membrane hyperpolarization of intact endothelial cells from rat aorta during acetylcholine stimulation. *Br J Pharmacol* **143**: 9–18.

Bondarenko A, Sagach V (2006). Na^+-K^+-ATPase is involved in the sustained ACh-induced hyperpolarization of endothelial cells from rat aorta. *Br J Pharmacol* **149**: 958–965.

Chen GF, Suzuki H (1990). Calcium dependency of the endothelium-dependent hyperpolarization in smooth muscle cells of the rabbit carotid artery. *J Physiol (Lond)* **421**: 521–534.

Corda D, Iurisci C, Berrie CP (2002). Biological activities and metabolism of the lysophosphoinositides and glycerophosphoinositols. *Biochim Biophys Acta* **1582**: 52–69.

Edgell CJ, McDonald CC, Graham JB (1983). Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci USA* **80**: 3734–3737.

Erdogan A, Schaefer MB, Kuhlmann CR, Most A, Hartmann M, Mayer K *et al*. (2007). Activation of Ca^{2+} -activated potassium channels is involved in lysophosphatidylcholine-induced monocyte adhesion to endothelial cells. *Atherosclerosis* **190**: 100–105.

Falasca M, Corda D (1994). Elevated levels and mitogenic activity of lysophosphatidylinositol in k-ras-transformed epithelial cells. *Eur J Biochem* **221**: 383–389.

Flygare J, Sander B (2008). The endocannabinoid system in cancer-potential therapeutic target? *Semin Cancer Biol* **18**: 176–189.

Frieden M, Graier WF (2000). Subplasmalemmal ryanodine-sensitive Ca^{2+} release contributes to Ca^{2+}-dependent K^+ channel activation in a human umbilical vein endothelial cell line. *J Physiol* **524** (Pt 3): 715–724.

Fukao M, Hattori Y, Kanno M, Sakuma I, Kitabatake A (1995). Evidence for selective inhibition by lysophosphatidylcholine of acetylcholine-induced endothelium-dependent hyperpolarization and relaxation in rat mesenteric artery. *Br J Pharmacol* **116**: 1541–1543.

Gardell SE, Dubin AE, Chun J (2006). Emerging medicinal roles for lysophospholipid signaling. *Trends Mol Med* **12**: 65–75.

Graier WF, Simecek S, Sturek M (1995). Cytochrome P450 mono-oxygenase-regulated signalling of Ca^{2+} entry in human and bovine endothelial cells. *J Physiol* **482** (Pt 2): 259–274.

Graier WF, Holzmann S, Hoebel BG, Kukovetz WR, Kostner GM (1996). Mechanisms of L-N^G nitroarginine/indomethacin-resistant relaxation in bovine and porcine coronary arteries. *Br J Pharmacol* **119**: 1177–1186.

Henstridge CM, Balenga N, Ford LA, Ross RA, Waldhoer M, Irving AJ (2008). The GPR55 ligand L-{alpha}-lysophosphatidylinositol promotes RhoA-dependent Ca^{2+} signaling and NFAT activation. *FASEB J* **23**: 183–193.

Hoebel BG, Kostner GM, Graier WF (1997). Activation of microsomal cytochrome P450 mono-oxygenase by Ca^{2+} store depletion and its contribution to Ca^{2+} entry in porcine aortic endothelial cells. *Br J Pharmacol* **121**: 1579–1588.

Hong SL, McLaughlin NJ, Tzeng CY, Patton G (1985). Prostacyclin synthesis and deacylation of phospholipids in human endothelial cells: comparison of thrombin, histamine and ionophore A23187. *Thromb Res* **38**: 1–10.

Howlett AC, Mukhopadhyay S (2000). Cellular signal transduction by anandamide and 2-arachidonoylglycerol. *Chem Phys Lipids* **108**: 53–70.

Iwamoto T, Uehara A, Nakamura TY, Imanaga I, Shigekawa M (1999). Chimeric analysis of Na^+/Ca^{2+} exchangers NCX1 and NCX3 reveals structural domains important for differential sensitivity to external Ni^{2+} or Li^+. *J Biol Chem* **274**: 23094–23102.

Jabr RI, Yamazaki J, Hume JR (2000). Lysophosphatidylcholine triggers intracellular calcium release and activation of non-selective cation channels in renal arterial smooth muscle cells. *Pflugers Arch* **439**: 495–500.

Kapur A, Zhao P, Sharir H, Bai Y, Caron MG, Barak LS *et al*. (2009). Atypical responsiveness of the orphan receptor GPR55 to cannabinoid ligands. *J Biol Chem* **284**: 29817–29827.

Kaya H, Patton GM, Hong SL (1989). Bradykinin-induced activation of phospholipase A2 is independent of the activation of polyphosphoinositide-hydrolyzing phospholipase C. *J Biol Chem* **264**: 4972–4977.

Kuhlmannm C, Lüdders D, Schaefer C, Most A, Backenköhler U, Neumann T *et al*. (2004). Lysophosphatidylcholine-induced modulation of Ca^{2+}-activated K^+ channels contributes to ROS-dependent proliferation of cultured human endothelial cells. *J Mol Cell Cardiol* **36**: 675–682.

Lauckner JE, Jensen JB, Chen HY, Lu HC, Hille B, Mackie K (2008). GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. *Proc Natl Acad Sci USA* **105**: 2699–2704.

Ledoux J, Bonev AD, Nelson MT (2008). Ca^{2+}-activated K^+ channels in murine endothelial cells: block by intracellular calcium and magnesium. *J Gen Physiol* **131**: 125–135.

McCarty MF (1999). Endothelial membrane potential regulates production of both nitric oxide and superoxide – a fundamental determinant of vascular health. *Med Hypotheses* **53**: 277–289.

Malli R, Frieden M, Osibow K, Graier WF (2003). Mitochondria efficiently buffer subplasmalemmal Ca^{2+} elevation during agonist stimulation. *J Biol Chem* **278**: 10807–10815.

Malli R, Frieden M, Hunkova M, Trenker M, Graier WF (2007). Ca^{2+} refilling of the endoplasmic reticulum is largely preserved albeit reduced Ca^{2+} entry in endothelial cells. *Cell Calcium* **41**: 63–76.

Meier SD, Kovalchuk Y, Rose CR (2006). Properties of the new fluorescent Na^+ indicator CoroNa Green: comparison with SBFI and confocal Na^+ imaging. *J Neurosci Methods* **155**: 251–259.

Murugesan G, Fox PL (1996). Role of lysophosphatidylcholine in the inhibition of endothelial cell motility by oxidized low density lipoprotein. *J Clin Invest* **97**: 2736–2744.

Nilius B, Droogmans G (2001). Ion channels and their functional role in vascular endothelium. *Physiol Rev* **81**: 1415–1459.

Ohta T, Imagawa T, Ito S (2008). Novel gating and sensitizing mechanism of capsaicin receptor (TRPV1): tonic inhibitory regulation of extracellular sodium through the external protonation sites on TRPV1. *J Biol Chem* **283**: 9377–9387.

Oishi K, Zheng B, Kuo JF (1990). Inhibition of Na,K-ATPase and sodium pump by protein kinase C regulators sphingosine, lysophosphatidylcholine, and oleic acid. *J Biol Chem* **265**: 70–75.

Oka S, Nakajima K, Yamashita A, Kishimoto S, Sugiura T (2007). Identification of GPR55 as a lysophosphatidylinositol receptor. *Biochem Biophys Res Commun* **362**: 928–934.

Oka S, Toshida T, Maruyama K, Nakajima K, Yamashita A, Sugiura T (2008). 2-Arachidonoyl-sn-glycero-3-phosphoinositol: a possible natural ligand for GPR55. *J Biochem* **145**: 13–20.

Paltauf-Doburzynska J, Posch K, Paltauf G, Graier WF (1998). Stealth ryanodine-sensitive Ca^{2+} release contributes to activity of capacitative Ca^{2+} entry and nitric oxide synthase in bovine endothelial cells. *J Physiol* **513** (Pt 2): 369–379.

Paltauf-Doburzynska J, Frieden M, Spitaler M, Graier WF (2000). Histamine-induced Ca^{2+} oscillations in a human endothelial cell line depend on transmembrane ion flux, ryanodine receptors and endoplasmic reticulum Ca^{2+}-ATPase. *J Physiol* **524** (Pt 3): 701–713.

Randall MD, Kendall DA (1998). Endocannabinoids: a new class of vasoactive substances. *Trends Pharmacol Sci* **19**: 55–58.

Ridgway ND, Byers DM, Cook HW, Storey MK (1999). Integration of phospholipid and sterol metabolism in mammalian cells. *Prog Lipid Res* **38**: 337–360.

Ross R (2009). The enigmatic pharmacology of GPR55. *Trends Pharmacol Sci* **30**: 156–163.

Ryberg E, Larsson N, Sjogren S, Hjorth S, Hermansson NO, Leonova J *et al*. (2007). The orphan receptor GPR55 is a novel cannabinoid receptor. *Br J Pharmacol* **152**: 1092–1101.

Shuttleworth TJ, Thompson JL, Mignen O (2004). ARC channels: a novel pathway for receptor-activated calcium entry. *Physiology (Bethesda)* **19**: 355–361.

Singaravelu K, Lohr C, Deitmer JW (2006). Regulation of store-operated calcium entry by calcium-independent phospholipase A_2 in rat cerebellar astrocytes. *J Neurosci* **26**: 9579–9592.

Smani T, Zakharov SI, Leno E, Csutora P, Trepakova ES, Bolotina VM (2003). Ca^{2+}-independent phospholipase A_2 is a novel determinant of store-operated Ca2+ entry. *J Biol Chem* **278**: 11909–11915.

Smani T, Zakharov SI, Csutora P, Leno E, Trepakova ES, Bolotina VM (2004). A novel mechanism for the store-operated calcium influx pathway. *Nat Cell Biol* **6**: 113–120.

Smani T, Domínguez-Rodríguez A, Hmadcha A, Calderón-Sánchez E, Horrillo-Ledesma A, Ordóñez A (2007). Role of Ca^{2+}-independent phospholipase A_2 and store-operated pathway in urocortin-induced vasodilatation of rat coronary artery. *Circ Res* **101**: 1194–1203.

Smyth SS, Cheng HY, Miriyala S, Panchatcharam M, Morris AJ (2008). Roles of lysophosphatidic acid in cardiovascular physiology and disease. *Biochim Biophys Acta* **1781**: 563–570.

Terasawa K, Nakajima T, Iida H, Iwasawa K, Oonuma H, Jo T *et al*. (2002). Nonselective cation currents regulate membrane potential of rabbit coronary arterial cell: modulation by lysophosphatidylcholine. *Circulation* **106**: 3111–3119.

Trenker M, Malli R, Fertschai I, Levak-Frank S, Graier WF (2007). Uncoupling proteins 2 and 3 are fundamental for mitochondrial Ca^{2+} uniport. *Nat Cell Biol* **9**: 445–452.

Waldeck-Weiermair M, Zoratti C, Osibow O, Balenga N, Goessnitzer E, Waldhoer M *et al*. (2008). Integrin-clustering enables endocannabinoid-induced Ca^{2+} signaling in endothelial cells via GPR55 by protection against CB_1-receptor triggered repression. *J Cell Sci* **121**: 1704–1717.

Xiao YJ, Schwartz B, Washington M, Kennedy A, Webster K, Belinson J *et al*. (2001). Electrospray ionization mass spectrometry analysis of lysophospholipids in human ascitic fluids: comparison of the lysophospholipid contents in malignant vs nonmalignant ascitic fluids. *Anal Biochem* **290**: 302–313.

Yin H, Chu A, Li W, Wang B, Shelton F, Otero F *et al*. (2009). Lipid G protein-coupled receptor ligand identification using beta-arrestin PathHunter assay. *J Biol Chem* **284**: 12328–12338.

Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sørgård M, Di Marzo V *et al*. (1999). Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400**: 452–457.