Characterization of calcium signals provoked by lysophosphatidylinositol in human microvascular endothelial cells

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Running title: Calcium signals by LPI in endothelial cells

Summary

The lipid molecule, lysophosphatidylinositol (LPI), is hypothesized to form part of a novel lipid signalling system that involves the G protein-coupled receptor GPR55 and distinct intracellular signalling cascades in endothelial cells. This work aimed to study the possible mechanisms involved in LPI-evoked cytosolic Ca^{2+} mobilization in human brain microvascular endothelial cells. Changes in intracellular Ca^{2+} concentrations were measured using cell population Ca^{2+} assay. LPI evoked biphasic elevation of intracellular calcium concentration, a rapid phase and a sustained phase. The rapid phase was attenuated by the inhibitor of PLC (U 73122), inhibitor of IP_3 receptors, 2-APB and the depletor of endoplasmic reticulum Ca^{2+} store, thapsigargin. The sustained phase, on the other hand, was enhanced by U 73122 and abolished by the RhoA kinase inhibitor, Y-27632. In conclusion, the Ca^{2+} signal evoked by LPI is characterized by a rapid phase of Ca^{2+} release from the endoplasmic reticulum, and requires activation of the PLC-IP_3 signalling pathway. The sustained phase mainly depends on RhoA kinase activation. LPI acts as novel lipid signalling molecule in endothelial cells, and elevation of cytosolic Ca^{2+} triggered by it may present an important intracellular message required in gene expression and controlling of vascular tone.

Keywords: lysophosphatidylinositol; GPR55; Calcium; PLC; RhoA kinase

1. Introduction

There is increasing evidence that long-chain lipids have a wide variety of actions that are exerted through G protein-coupled receptors (Makide et al., 2014). The bioactive lipid, lysophosphatidylinositol (LPI) is now accepted as an endogenous ligand of G protein-couple receptor GPR55. When LPI tested in systems expressing GPR55, it initiated responses, which were either absent in untransfected cells, diminished by siRNA knockdown of GPR55 or enhanced by GPR55 overexpression (Bondarenko et al., 2010; Ford et al., 2010; Henstridge et al., 2009; Henstridge et al., 2010; Oka et al., 2010; Oka et al., 2007; Waldeck-Weiermair et al., 2008). For instance, in HEK293 cells expressing a human GPR55 receptor, LPI induced Ca^{2+} mobilization (Henstridge et al., 2009), caused phosphorylation of extracellular signal-regulated kinases (Oka et al., 2007; Oka et al., 2009), and activated RhoA and nuclear factor of activated-T cells (Henstridge et al., 2009). Additionally, LPI initiated Ca^{2+} mobilization in mice dorsal root ganglion neurons (Lauckner et al., 2008) and human endothelial cells naturally expressing GPR55 (Bondarenko et al., 2010; Waldeck-Weiermair et al., 2008). In human umbilical vein endothelial cells (HUVEC), LPI induced both GPR55-dependent and -independent signals (Bondarenko et al., 2010; Waldeck-Weiermair et al., 2008), and acted as an intracellular messenger modulating Ca^{2+}-activated K^+ channels (Bondarenko et al., 2011a). The lipid also induced wound healing in primary human lung microvascular endothelial cells and causes platelets aggregation (Kargl et al., 2013).

With increasing evidence of vascular role of GPR55 and with lack of clarity of LPI action in vasculature, this study aims to further characterize the cellular action of LPI in endothelial cells. The intracellular Ca^{2+} signals in response to LPI in human brain microvascular endothelial cells were investigated.

2. Methods

2.1 Cell culture

The human immortalised brain endothelial cell line (hCMEC/D3) was provided by Dr Margery Barrand, Department of Pharmacology, University of Cambridge. The cell line was originally donated by Dr Pierre Couraud, INSERM, rue de Tolbiac, Paris, France, and line was established from isolated human microvascular brain endothelial cells (Weksler et al., 2005). The cells were grown on surfaces coated with 0.5% bovine gelatine (Sigma), and were maintained in endothelial cell basal medium-2 (Lonza, Cologne, Germany) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen) together with 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM L-glutamine, 80 µg ml^{-1} heparin, 5 µg ml^{-1} ascorbic acid (all from Sigma), and 75 µg ml^{-1} endothelial growth supplement (First Link, Birmingham). The cells were incubated at 37^{o}C in a humidified 5% CO_2 atmosphere, and passaged when they reached ~90-95% confluence. Experiments were conducted on cultures between passages 36 and 40.

2.2 Real-time PCR

Total RNA was extracted from the hCMEC/D3 cells using TRIZOL® (Invitrogen) as explained in the manufacturer's protocol. Briefly, the cells at ~90% confluence in T75 flasks were homogenized in 1 ml TRIZOL® and total RNA was separated and precipitated and used to produce cDNA. Briefly, total RNA was subjected to DNase digestion before proceeding to cDNA synthesis using SuperScript™ II reverse transcriptase (Invitrogen) to synthesize the first strand cDNA. A quantitative polymerase chain reaction (qPCR) was carried out using the SensiMix™ SYBR & Fluorescein Kit (Bioline, London).

2.3 Population Ca^{2+} imaging

HCMEC/D3 cells were seeded onto 0.5% gelatine-coated wells of 96-well black plates (Greiner Bio-One Ltd, Stonehouse, Gloucestershire), and used for experiments 24 h later. Confluent cultures were first washed twice with phosphate-buffered saline (PBS, Invitrogen) and then loaded with 2 µM fluo-4 acetoxymethyl ester (Invitrogen). This was achieved by incubating the cells in HEPES-buffered saline (HBS; containing in mM: NaCl 135, KCl 5.9, $MgCl_2$ 1.2, HEPES 11.6, glucose 11.5, $CaCl_2$ 1.5, pH 7.3) supplemented with 2.5 mM probenecid (Sigma) and fluo-4 acetoxymethyl ester for 1 h at 20˚C. The cells were then washed with HBS and further incubated for 30 min to allow de-esterification of the dye before being used for experiments. All experiments were performed in HBS (or nominally Ca^{2+}-free HBS) at 20°C. The plate containing fluo-4-loaded cells was mounted in a FlexStation III (MDS Analytical Technologies, Wokingham, Surrey) to measure changes in fluorescence (excitation at 485 nm;

emission at 525 nm). Drugs were prepared in another 96-well plate (Greiner Bio-One Ltd), and were automatically added to the loaded cells. The machine was programmed to allow for six simultaneous readings from each well at intervals of 1.52 s. At the end of each experiment, a solution containing 0.05% Triton X-100 (Sigma) and 10 mM $CaCl_2$ was added to the cells in order to determine the fluorescence of Ca^{2+}-saturated indicator (F_{max}). Background fluorescence (F_{min}), where cells containing only Ca^{2+}-free indicator, was measured in parallel wells treated with a combination of 0.05% Triton X-100 and 10 mM BAPTA (Molekula, Gillingham, Dorset). This background was subtracted from all measurements. Data were recorded on SoftMax Pro version 5.4 (Govindan et al., 2010). Calcium Responses to LPI were recorded for a period of 5 minutes. Antagonists were incubated with cells for 10 min before being exposed to LPI.

2.4 Data and statistical analysis

In the population Ca^{2+} assay, both basal and peak cytosolic free Ca^{2+} concentrations were calculated using the formula:

$$[Ca^{2+}]_i = K_D F/(F_{max} - F)$$

where K_D is the Ca^{2+} dissociation constant of fluo-4 (345 nM; (Gee et al., 2000), F is the corrected fluorescence (for basal and peak Ca^{2+} levels) and F_{max} is the fluorescence of the Ca^{2+}-saturated indicator. Data are expressed as $\Delta[Ca^{2+}]_i$, which indicates the difference in cytosolic Ca^{2+} concentration between basal and maximum release in response to a drug. In single cell Ca^{2+} imaging experiments, data are expressed as Δ ratio (F_{340}/F_{380}) which indicates the difference in 340 to 380 nm fluorescence ratios between basal and peak Ca^{2+}. *n* represents number of cells from at least 2 independent isolations.

In experiments where mRNA expression was examined, the authenticity of each product was assessed by melting-curve analysis and the results were analyzed using the “comparative quantitation” feature of the Rotor-Gene software. Concentration-response curves were analyzed using 2-way ANOVA, and in those experiments where more than two groups were compared, statistical analysis was performed using one-way ANOVA followed by Bonferroni’s *post hoc* test. A *P* value of less than 0.05 was taken as statistically significant. Data were analyzed using GraphPad Prism (GraphPad, San Diego, CA, USA).

2.5 Drugs

L-α-Lysophosphatidylinositol, histamine (all from the Sigma Chemical Company, Poole, Dorset) and Y-27632 (Tocris Cookson, Bristol) were dissolved in distilled water. 2-aminoethoxydiphenyl borate (2-APB; from Tocris) was dissolved in 100% ethanol. U 73122 (Tocris), U 73343 (Sigma), and thapsigargin (Sigma) were dissolved in 100% dimethyl sulfoxide (DMSO). All solutions were prepared on the day of the experiment.

3. Results

3.1 Expression profile of GPR55, CB_1 and CB_2 receptors in hCMEC/D3 cell line

Expression of GPR55 and the cannabinoid receptors CB_1 and CB_2 in the hCMEC/D3 cell line was examined using qPCR. Analyses of the melting curves revealed single specific peaks beyond 80˚C. Low levels of mRNA transcripts for GPR55, CB_1 and CB_2 receptors were detected in this cell line, and all the receptors were approximately equally expressed when compared to the reference gene β-actin (Fig. 1).

3.2 Effect of histamine on $[Ca^{2+}]_i$ mobilization in hCMEC/D3 cells

In this study, the effect of histamine on cytosolic Ca^{2+} mobilization was first tested before proceeding to examine the actions of LPI. In endothelial cells, histamine acts on H_1-histamine receptors that are linked to the $G_{\alpha q}$-PLC-IP_3 pathway, resulting in elevation of $[Ca^{2+}]_i$ (Smit et al., 1999). In the population Ca^{2+} assay when extracellular Ca^{2+} was present, histamine (10 µM) provoked a rapid rise in $[Ca^{2+}]_i$ which was characterized by a peak (218 ± 30 nM) followed by a sustained phase(Fig. 2A). This latter phase disappeared when Ca^{2+}-free buffer was used, indicating the need for Ca^{2+} entry (Fig. 2B). The downstream signalling pathway involved in the elevation of $[Ca^{2+}]_i$ was then investigated using enzyme inhibitors and receptor antagonists. As shown in Figure 2B and C, in nominally Ca^{2+}-free HBS, the rise in $[Ca^{2+}]_i$ in response to histamine was abolished by depletion of intracellular Ca^{2+} stores by thapsigargin (1 µM), inhibition of PLC by U 73122 (10 µM) and antagonism of IP_3 receptors by 2-APB (100 µM). The selective inhibitor of the RhoA-specific kinase p160ROCK, Y-27632 (50 µM) had no effect on the histamine response.

3.3 Characterization of LPI-evoked Ca^{2+} transients in hCMEC/D3 cells

Figure 3 shows that, using the population Ca^{2+} assay, LPI elicited a biphasic, concentration-dependent, elevation of $[Ca^{2+}]_i$ in hCMEC/D3 cells which was independent of extracellular Ca^{2+}. The initial phase was rapid (~8 s following LPI application) and transient, whereas the late phase was characterized by a slowly developing, sustained, elevation of $[Ca^{2+}]_i$ following the rapid phase, which was only observed at higher concentrations (above 10 µM).

3.4 Effects of thapsigargin, 2-APB, U 73122, U 73343 and Y-27632 on the early phase of the Ca^{2+} response

Since LPI induced a rise in $[Ca^{2+}]_i$in which there were two peaks, the signalling pathway involved in each was studied independently. Pretreatment with thapsigargin (1 µM, 15 min incubation) attenuated the response to LPI (Fig. 4A) and the IP_3 receptor antagonist, 2-APB (100 µM, 10-min incubation) also abolished the response (Fig. 4B). The PLC inhibitor, U 73122 (10 µM, 10-min incubation) also abolished the rapid rise in $[Ca^{2+}]_i$ in response to LPI, whereas its inactive analogue, U 73343 (10 µM) had no effect (Fig. 5). Treating the cells with 50 µM Y-27632 (20-min incubation) resulted in a significant reduction in the elevation of $[Ca^{2+}]_i$ by LPI (Fig. 6).

3.5 Effect of thapsigargin, 2-APB, U 73122 and Y-27632 on the sustained Ca^{2+} response to LPI

As mentioned above, the slowly developing, sustained, phase of the cytosolic Ca^{2+} response to LPI was observed at higher concentrations of the lysolipid. Interestingly, neither thapsigargin nor 2-APB, used at concentrations the same concentration as above, had any noticeable effect on this phase in responses to 30 µM LPI (Fig. 7). Even more interestingly, exposing the cells to 10 µM U 73122 for 10 min produced a prominent concentration-dependent potentiation of the slow phase of Ca^{2+} signals evoked by LPI (Fig. 8) On the other hand, Y-27632 (50 µM) abolished the sustained phase (Fig. 9).

4. Discussion

In endothelial cells, recent studies have shown that LPI induces intracellular Ca^{2+} mobilization by activation of GPR55 (Bondarenko et al., 2010; Waldeck-Weiermair et al., 2008). The present study extends these observations to demonstrate that LPI initiates concentration-dependent cytosolic Ca^{2+} mobilization in human brain microvascular endothelial cells (hCMEC/D3). The Ca^{2+} signal evoked by LPI is characterized by a rapid phase of Ca^{2+} release from the endoplasmic reticulum, and requires activation of the PLC-IP_3 signalling pathway and RhoA-dependent kinases. LPI also mediates a sustained phase of elevation of $[Ca^{2+}]_i$ which entirely depends on RhoA kinase activation.

In hCMEC/D3 endothelial cells, LPI, concentration-dependently, stimulated biphasic elevation of cytosolic Ca^{2+}. The rapid phase was characterized by a modest elevation of $[Ca^{2+}]_i$ (~40 nM at 30 µM LPI). Higher concentrations of LPI (> 30 µM) were not tested due to the possibility of micelle formation beyond this concentration (Bondarenko et al., 2011b). Since there was no obvious maximal effect at the highest concentration used, the EC_{50} of LPI could not be determined in this population assay. The LPI-initiated rapid elevation of $[Ca^{2+}]_i$ was due to release from intracellular stores, as thapsigargin, the inhibitor of SERCA, attenuated the response. This was further confirmed by abolition of the signal by antagonism of the IP_3 receptor using 2-APB, suggesting release from endoplasmic reticulum. Moreover, to test if PLC was involved in this process, the cells were treated with U 73122, an inhibitor of PLC, at a concentration previously reported to inhibit the enzyme (Bleasdale et al., 1990). Indeed, exposing the cells to U 73122 for 15 min diminished the Ca^{2+} signals to LPI, indicating a role for PLC. Thus, the rapid elevation of $[Ca^{2+}]_i$ is a receptor-initiated response, and involves activation of PLC-IP_3 pathway. In human endothelial cells, LPI, in addition to reported receptor-independent effects (including activation of nonselective cation channels, inhibition of Na^+-K^+ ATPase, and bidirectional modulation of large- and intermediated-conductance Ca^{2+}-activated K^+ channels) (Bondarenko et al., 2010; Bondarenko et al., 2011a; b), activates signalling through GPR55 (Bondarenko et al., 2010; Waldeck-Weiermair et al., 2008). There is an increasing body of evidence suggesting that GPR55 signals through $G_{\alpha q}$ linked to

PLC-IP_3 and $G_{\alpha12/13}$ linked to RhoA. GPR55 is possibly involved in the rapid phase of $[Ca^{2+}]_i$ stimulated by LPI in hCMEC/D3 cells, as its mRNA transcript was detected in this cell line. In order to further investigate this, the involvement of RhoA- dependent kinases was examined. Indeed, a selective inhibitor of RhoA-specific kinase (p160ROCK), Y-27632 significantly reduced the response, pointing to a likely role of this kinase. RhoA-dependent Ca^{2+} signalling through GPR55 has recently been suggested (Henstridge et al., 2009). Overall, these findings suggest that LPI, by activation of GPR55, initiates Ca^{2+} transients through both PLC-IP_3 and RhoA pathways. Further studies will be required to determine if the response is mediated by dual signalling through both $G_{\alpha q}$ and $G_{\alpha 13}$.

As noted previously, LPI, in addition to inducting a rapid rise in $[Ca^{2+}]_i$, stimulated a sustained elevation of cytosolic Ca^{2+}, although this was seen only at higher concentrations. Study of this phase was interesting. Since the response was observed in Ca^{2+}-free extracellular buffer, this may suggest an intracellular mechanism of Ca^{2+} release. However, depletion of Ca^{2+} stores by thapsigargin and antagonism of the IP_3 receptor by 2-APB did not inhibit the response, indicating Ca^{2+} stores other than those in the endoplasmic reticulum might be involved in the sustained elevation of $[Ca^{2+}]_I$ caused by LPI. The contribution of PLC to this response was also investigated with the commonly used inhibitor U 73122. Surprisingly, when the cells were pretreated with the inhibitor, the LPI-induced sustained elevation of cytosolic Ca^{2+} was greatly enhanced in a concentration-dependent manner, suggesting involvement of PLC. In fact, a very recent study reported that U 73122, in addition to inhibition of some isoforms of PLC, possesses stimulatory activity on others (Klein et al., 2011). It was concluded that, at 10 μM, it increased the activity of human PLC isoforms β2, β3 and γ1, whereas the δ1 isoform was not affected. It was proposed that U 73122 binds covalently to PLC *via* cysteine residues leading to an increase in the affinity of the protein for cell membranes. One or more molecules of U 73122 may serve as lipid anchors for the modified PLC, allowing it to dock within the cell membrane in close proximity to substrate, thus leading to increased catalytic activity. Therefore, it is possible that LPI binding to a receptor (or by itself within the cell) activates one of the PLC isoforms that are activated by U 73122 leading to the observed enhanced elevation of cytosolic Ca^{2+}. As the LPI-initiated sustained elevation in $[Ca^{2+}]_i$ did not require activation of the IP_3 receptors, and was not sensitive to depletion of thapsigargin-sensitive stores, the role of PLC in cytosolic release of Ca^{2+} in this case remains unclear. It may suggest activation of other Ca^{2+} stores sensitive to PLC but not IP_3. One possible suggestion would be the mitochondria (De Marchi et al., 2014 & Park et al., 2014), however, further studied will be required to confirm this. In the current study, inhibition of RhoA-dependent kinase by Y-27632 attenuated the sustained phase of the LPI-induced $[Ca^{2+}]_i$ release, suggesting a possible role for RhoA kinases. Whether this effect is a receptor-mediated or not will require further studies to determine. In a recent work on LPI and endothelial cells (Al Suleimani & Hiley, 2015), similar findings were noticed in mesenteric endothelial cells. It was suggested that PLCε (which is regulated by RhoA), is the possible target in this phase. Further studies by using specific inhibitors of this PLC isoform will confirm this. Moreover, whether or not p160ROCK has direct regulatory activity on PLC, or indirectly through RhoA, is open to investigation. At least, this late phase of the Ca^{2+} response seems to require activation of both PLC and RhoA.

In the human umbilical vein endothelial cell line, EA.hy926, LPI induces GPR55-dependent and -independent elevation of cytosolic Ca^{2+} (Bondarenko et al., 2010). The receptor-dependent effect is sensitive to PLC inhibition while the receptor-independent effects involve activation of non-selective cation channels and inhibition of the Na^+/K^+-ATPase. The current study agrees with these findings in that LPI produced a biphasic effect on cytosolic Ca^{2+} elevation that was characterized by an initial rapid peak, most probably mediated by GPR55, and a sustained phase which could be a GPR55-independent effect. In HEK293 cells expressing GPR55, LPI stimulates elevation of $[Ca^{2+}]_i$ that is oscillatory in nature (Henstridge et al., 2009). In our very recent published study (Al Suleimani & Hiley, 2015) we have shown that LPI induced endothelium-dependent vasorelaxation of rat resistance mesenteric arteries and that the effect was independent of activation of guanylyl cyclase and cyclooxygenase metabolites, but largely involved activation of Ca^{2+}-activated K^+ channels.

Overall, the experiments reported here on human brain microvascular endothelial cells show that LPI act as novel lipid signalling molecule, and elevation of cytosolic Ca^{2+} triggered by it may present an important intracellular message required in gene expression and controlling of vascular tone.

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LEGENDS

Fig. 1. Expression of mRNA transcripts for GPR55, CB_1 and CB_2 receptors in the hCMEC/D3 endothelial cell line. (A) & (B) Representative real-time PCR measurements of the mRNA levels for the reference gene β-actin, GPR55, and the CB_1 and CB_2 receptors. Each curve is an average of duplicates from samples obtained from three different cell passages. The dashed lines represent samples where reverse-transcriptase (RT) was omitted, showing no specific amplification. (C) mRNA expression levels. Overexpression of human GPR55 in HEK293 cells (oxpGPR55) is shown as a positive control. Values are presented as mean expression relative to β-actin and vertical lines indicate the s.e.mean.

Fig. 2. Ca^{2+} signals evoked by histamine in hCMEC/D3 endothelial cells. Typical results from populations of the cells stimulated with histamine either in Ca^{2+}-containing buffer (A) or in Ca^{2+}-free buffer in the presence of thapsigargin, U 73122, 2-APB or Y-27632 (B). The results shown are means from three wells on a single plate and are typical of results from three independent plates from at least 3 different cell passages. (C) Histogram representing the peak $[Ca^{2+}]_i$ rise to histamine alone or in the presence of the enzyme inhibitors or the IP_3 receptor antagonist. *** represents statistical significance compared to the control group ($P < 0.001$). Values are means ± s.e.mean of triplicates obtained from 3 different cell passages.

Fig. 3. Ca^{2+} signals evoked by LPI in hCMEC/D3 endothelial cells. (A) Typical results from cell populations stimulated with 30 µM LPI in Ca^{2+}-free buffer, showing the biphasic $[Ca^{2+}]_i$ response. Results are means ± s.e.mean from three wells on a single plate. (B) Concentration-response relationship for phase 1 of the LPI-stimulated elevation of $[Ca^{2+}]_i$ in the presence or absence of extracellular Ca^{2+}. The effect of the vehicle (negative control) is also shown. (C) Effect of two concentrations of LPI on phase 2 of the elevation of the $[Ca^{2+}]_i$. Data from B & C are means ± s.e.mean of triplicates obtained from 3 different cell passages. (D) Recordings from populations of the cells each obtained from a single well showing the concentration-dependent effect of LPI on phase 1 of the elevation of $[Ca^{2+}]_i$. Each well of the 96-well plate was exposed to only a single concentration of LPI.

Fig. 4. Ca^{2+} signals evoked by LPI in hCMEC/D3 endothelial cells. Responses were initiated by LPI either alone or in the presence of thapsigargin (A) or 2-APB (B). Data are means of triplicates obtained from 3 different cell passages and vertical lines represent the s.e.mean. $[Ca^{2+}]_i$ responses were obtained in nominally Ca^{2+}-free buffer. Each well of the 96-well plate was exposed to only a single concentration of LPI.

Fig. 5. Ca^{2+} signals evoked by LPI in hCMEC/D3 endothelial cells. Responses were initiated by LPI alone or in the presence of either U 73122 or U 73343 (A). Data are means of triplicates obtained from 3 different cell passages and vertical lines represent the s.e.mean. (B) Representative recording from populations of the cells showing the abolition of Ca^{2+} signals to LPI by U 73122. Responses were recorded in nominally Ca^{2+}-free buffer. Each well of the 96-well plate was exposed to only a single concentration of LPI.

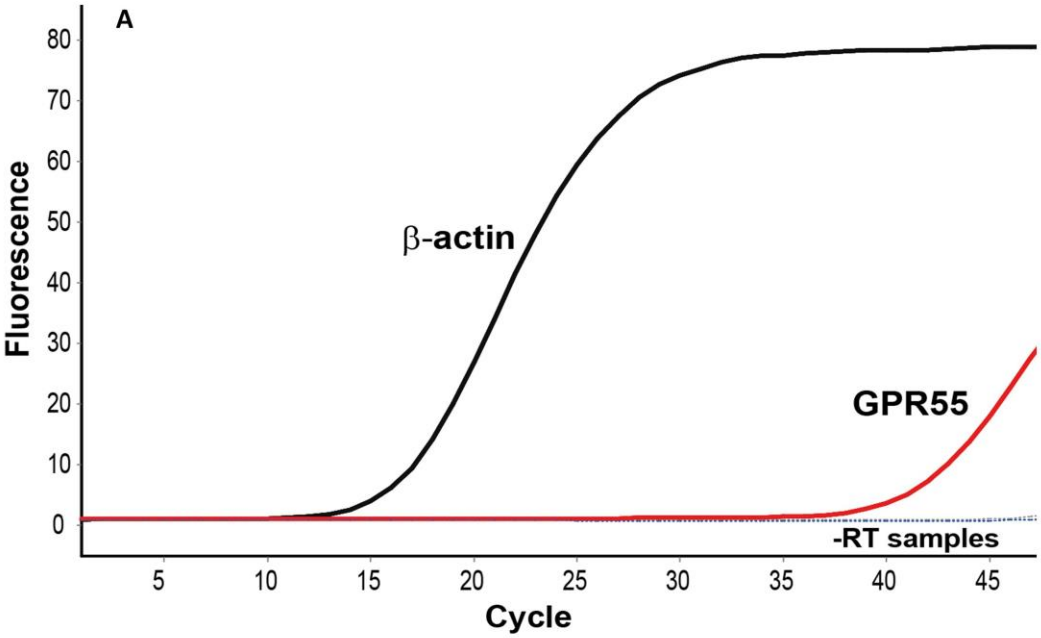
Fig. 6. Ca^{2+} signals evoked by LPI in hCMEC/D3 endothelial cells. Responses were initiated by LPI either alone or in the presence of Y-27632. Data are means of triplicates obtained from 3 different cell passages and vertical lines represent the s.e.mean. $[Ca^{2+}]_i$ responses were obtained in nominally Ca^{2+}-free buffer. Each well of the 96-well plate was exposed to only a single concentration of LPI.

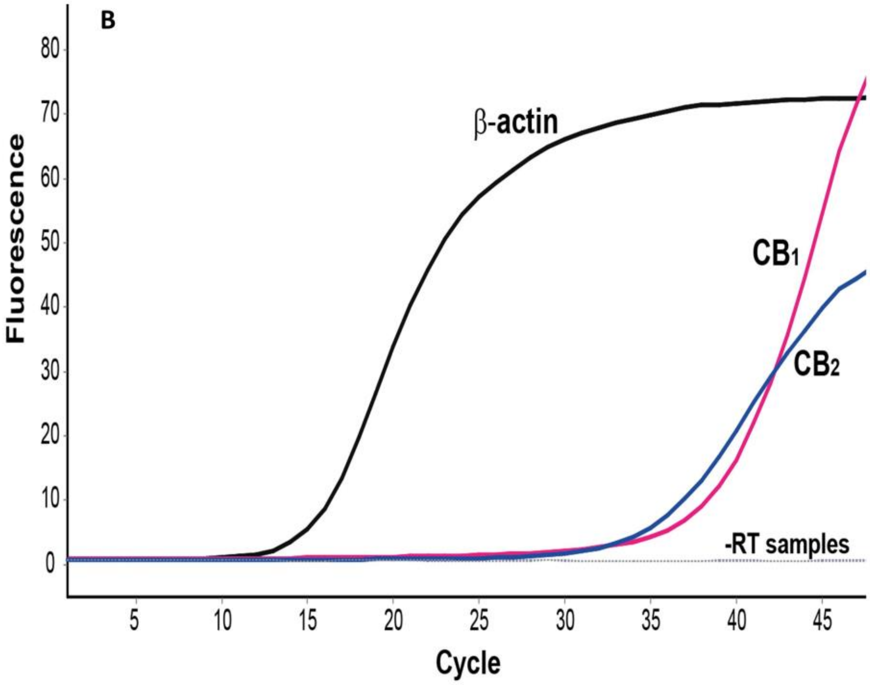
Fig. 7. LPI-evoked sustained elevation of $[Ca^{2+}]_i$. in hCMEC/D3 endothelial cells. Responses were initiated by LPI either alone or in the presence of thapsigargin or 2-APB. Data are means of triplicates obtained from 3 different cell passages and vertical lines represent the s.e.mean. $[Ca^{2+}]_i$ responses were obtained in nominally Ca^{2+}-free buffer. Each well of the 96-well plate was exposed to only a single concentration of LPI.

Fig. 8. LPI-evoked sustained elevation of $[Ca^{2+}]_i$ in hCMEC/D3 endothelial cells. Responses were initiated by LPI alone or in the presence of U 73122 (A). Data are means of triplicates obtained from 3 different cell passages and vertical lines represent the s.e.mean. (B) Typical results from cell populations stimulated with LPI showing enhancement of the sustained elevation of $[Ca^{2+}]_i$ by U 73122. Results are means ± s.e.mean from three wells on a single plate. Ca^{2+} signals were recorded in nominally Ca^{2+}-free buffer. Each well of the 96-well plate was exposed to only a single concentration of LPI.

Fig. 9. Sustained phase of the Ca^{2+} signals evoked by LPI in hCMEC/D3 endothalial cells. Responses were initiated by LPI either alone or in the presence of Y-27632 (A). Data are means of triplicates obtained from 3 different cell passages and vertical lines represent the s.e.mean. (B) Typical recording from cell populations showing the attenuation of the sustained elevation of [Ca^{2+}]$_i$ to LPI by Y-27632. Responses were recorded in nominally Ca^{2+}-free buffer. Each well of the 96-well plate was exposed to only a single concentration of LPI.

Figure 1


A
Fluorescence
80
70
60
50
40
30
20
10
0
β-actin
GPR55
-RT samples
5
10
15
20
25
30
35
40
45
Cycle


B
β-actin
CB1
CB2
-RT samples
Fluorescence
Cycle
0
10
20
30
40
50
60
70
80
5
10
15
20
25
30
35
40
45

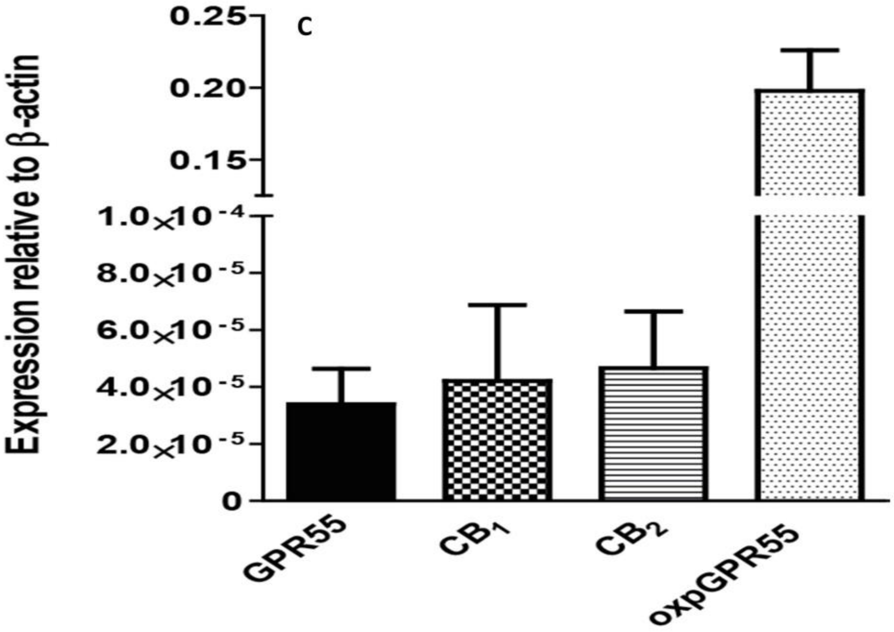
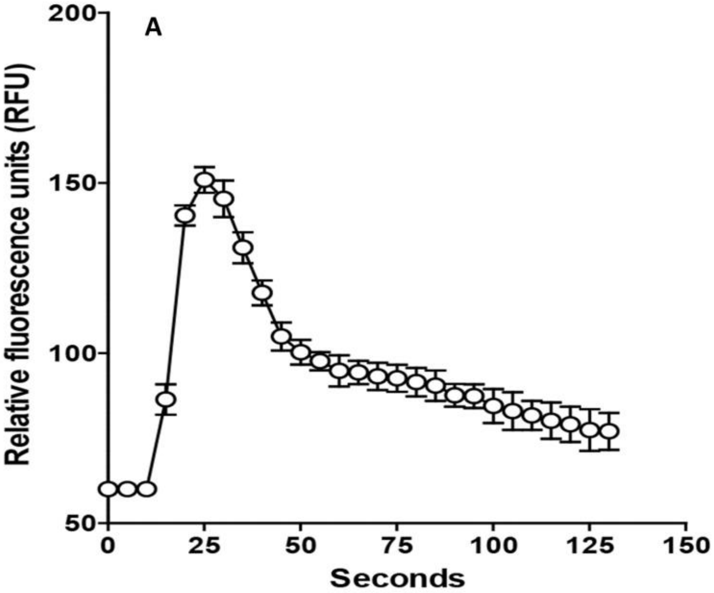
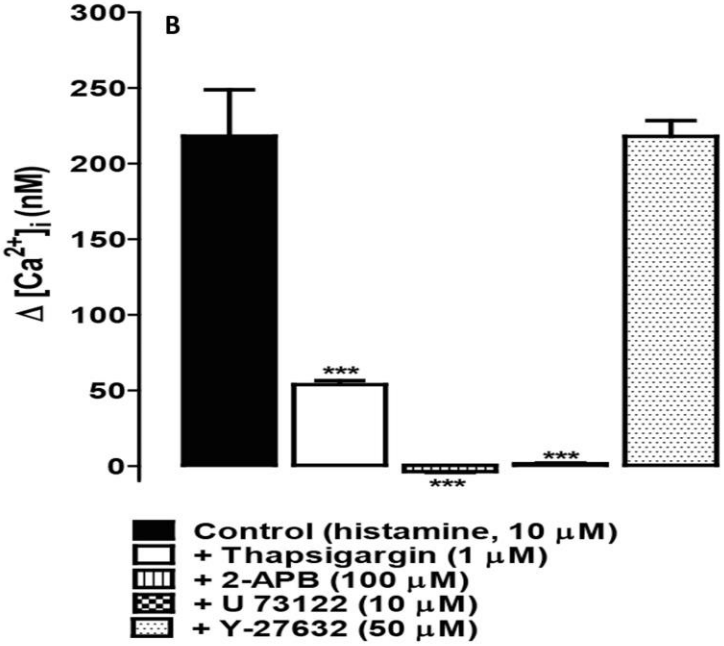
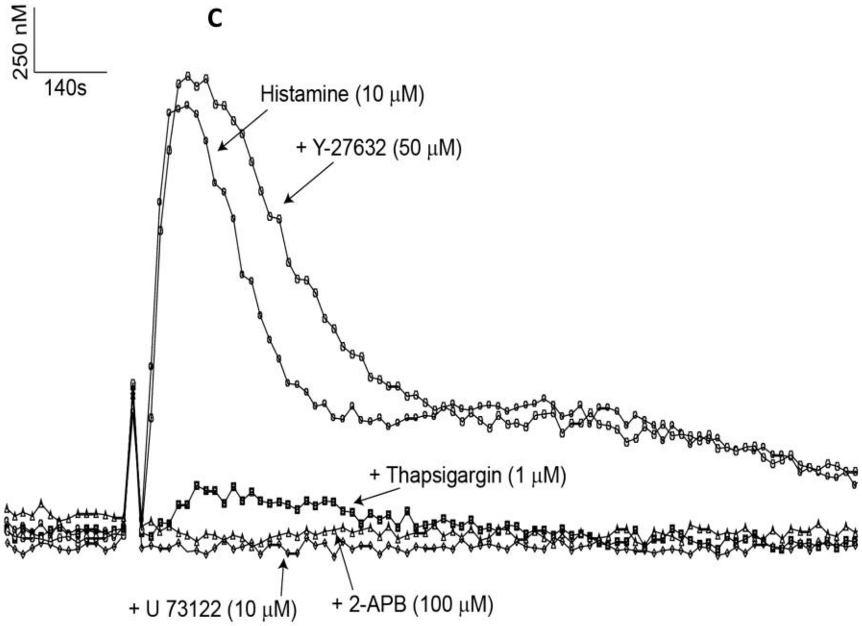
c
Expression relative to β-actin
0.25
0.20
0.15
1.0×10⁻⁴
8.0×10⁻⁵
6.0×10⁻⁵
4.0×10⁻⁵
2.0×10⁻⁵
0
GPR55
CB1
CB2
oxpGPR55

Figure 2

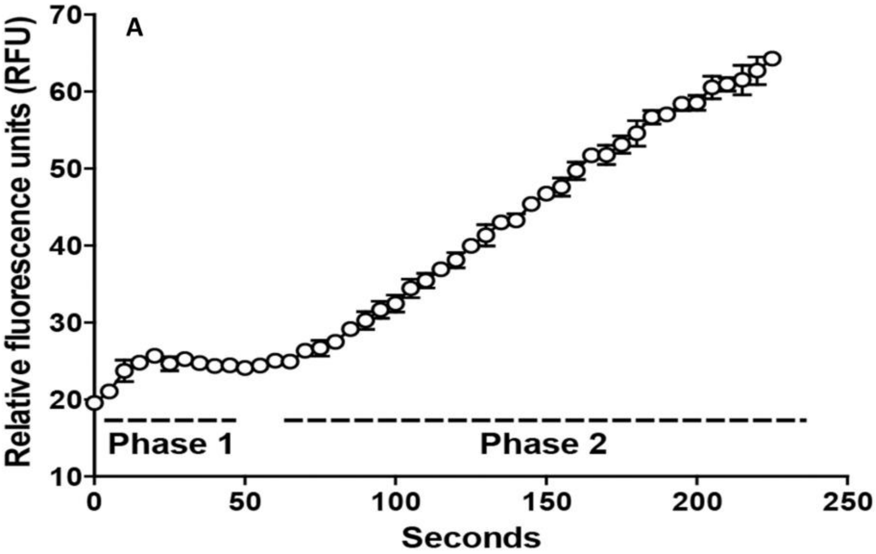

A
Relative fluorescence units (RFU)
200
150
100
50
0
25
50
75
100
125
150
Seconds

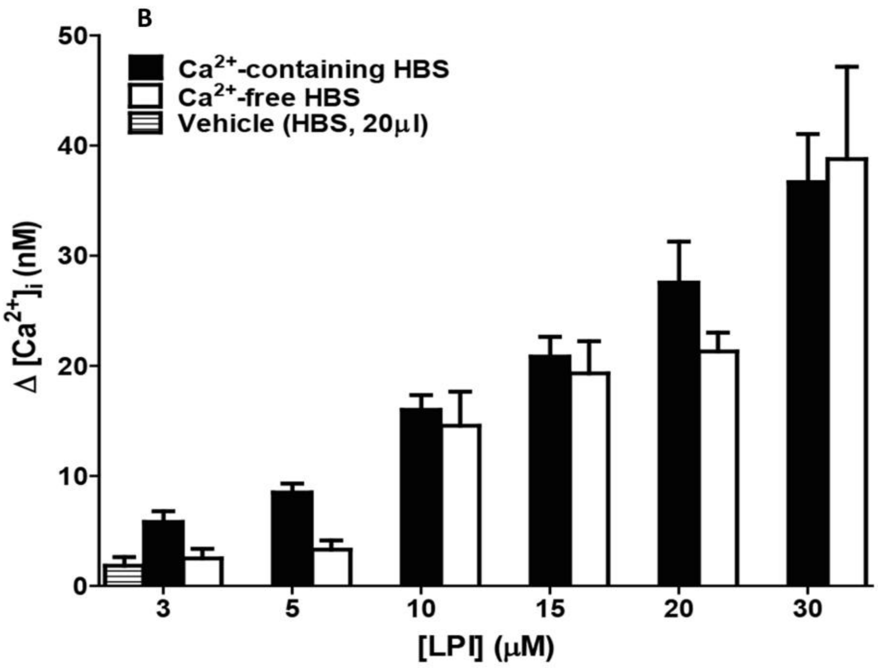
B
300
250
200
150
100
50
0
Δ [Ca2+]i (nM)
***
***
***
Control (histamine, 10 μM)
+ Thapsigargin (1 μM)
+ 2-APB (100 μM)
+ U 73122 (10 μM)
+ Y-27632 (50 μM)

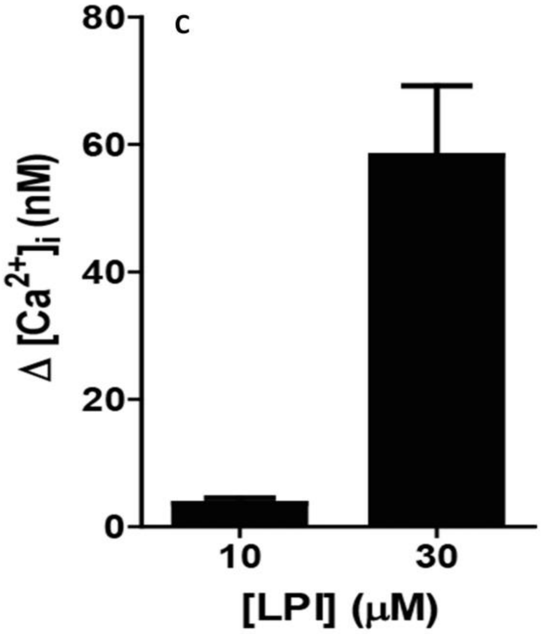


C
250 nM
140s
Histamine (10 μM)
+ Y-27632 (50 μM)
+ Thapsigargin (1 μM)
+ U 73122 (10 μM)
+ 2-APB (100 μM)

Figure 3


A
Relative fluorescence units (RFU)
70
60
50
40
30
20
10
0
50
100
150
200
250
Seconds
Phase 1
Phase 2


B
Ca2+-containing HBS
Ca2+-free HBS
Vehicle (HBS, 20µl)
Δ [Ca2+]i (nM)
50
40
30
20
10
0
3
5
10
15
20
30
[LPI] (µM)


c
Δ [Ca^{2+}]$_i$ (nM)
0
20
40
60
80
10
30
[LPI] (μM)

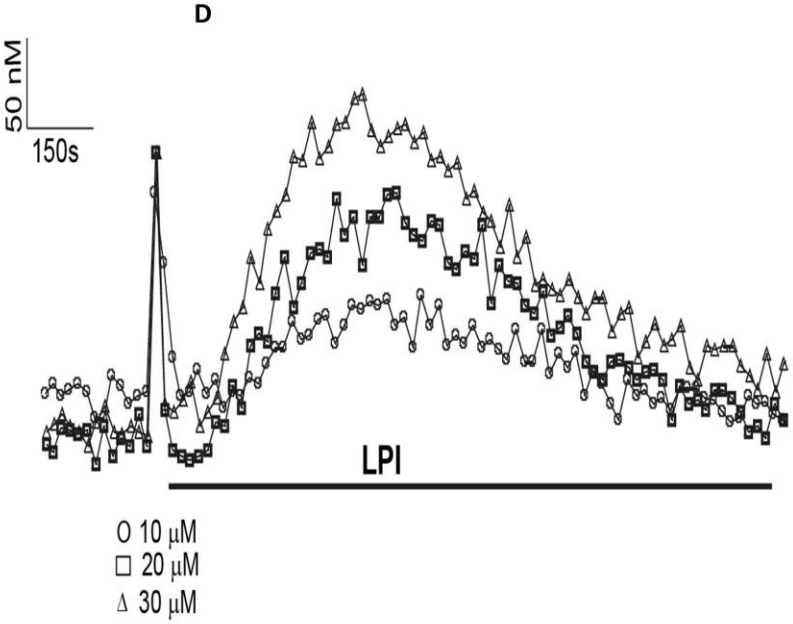
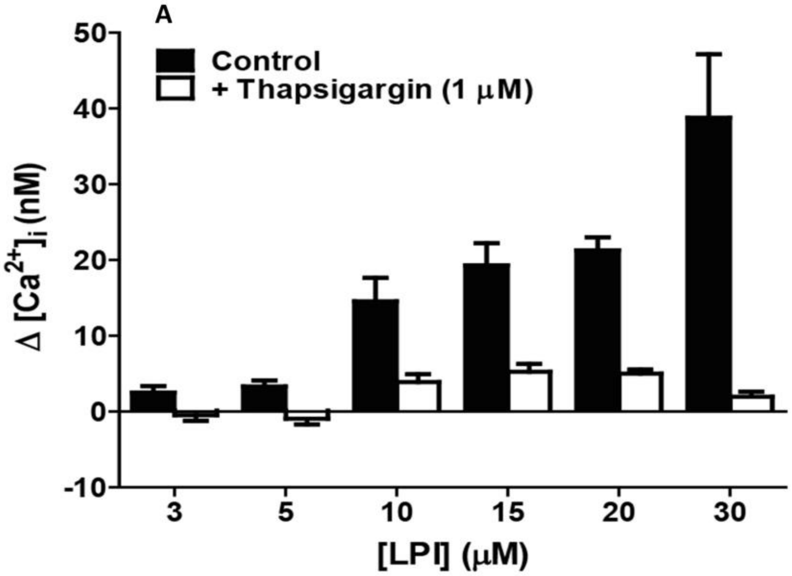

D
50 nM
150s
LPI
○ 10 μM
□ 20 μM
Δ 30 μM

Figure 4


A
Control
+ Thapsigargin (1 μM)
Δ [Ca2+]i (nM)
50
40
30
20
10
0
-10
3
5
10
15
20
30
[LPI] (μM)

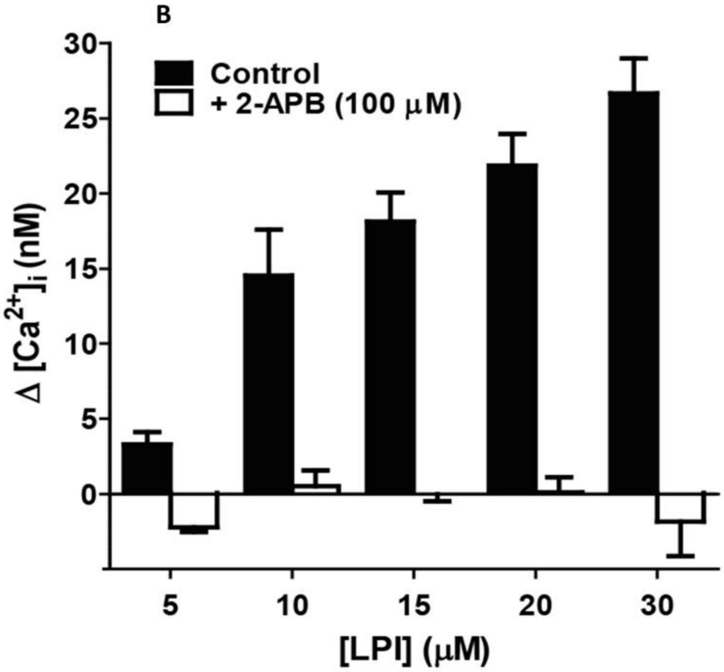
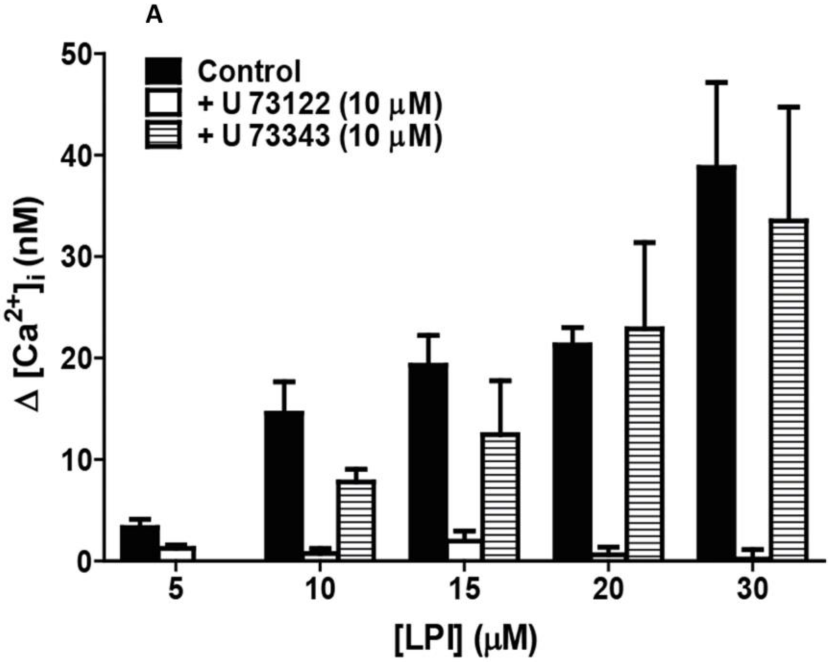

B
Control
+ 2-APB (100 μM)
Δ [Ca2+]i (nM)
30
25
20
15
10
5
0
5
10
15
20
30
[LPI] (μM)

Figure 5


A
Control
+ U 73122 (10 μM)
+ U 73343 (10 μM)
Δ [Ca2+]i (nM)
0
10
20
30
40
50
5
10
15
20
30
[LPI] (μM)

B

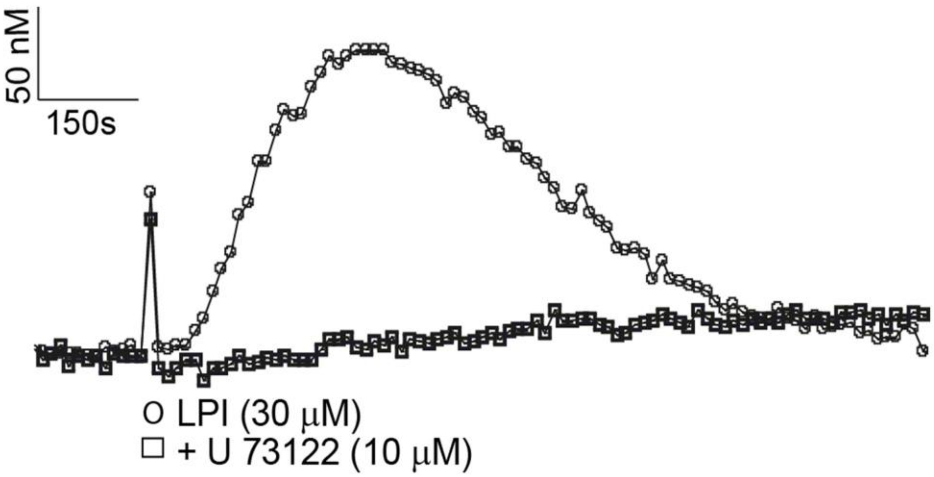


Figure 6

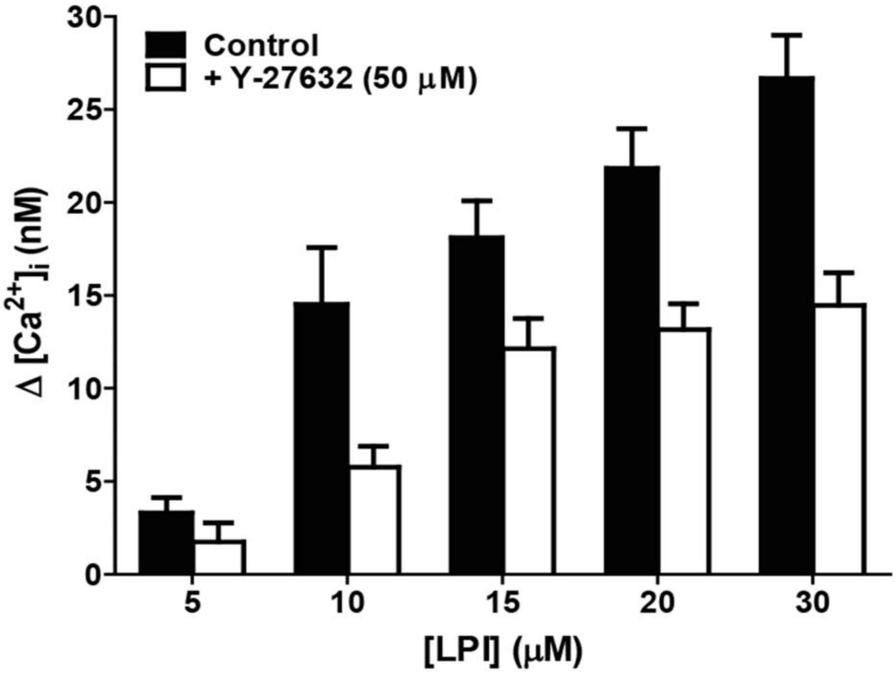
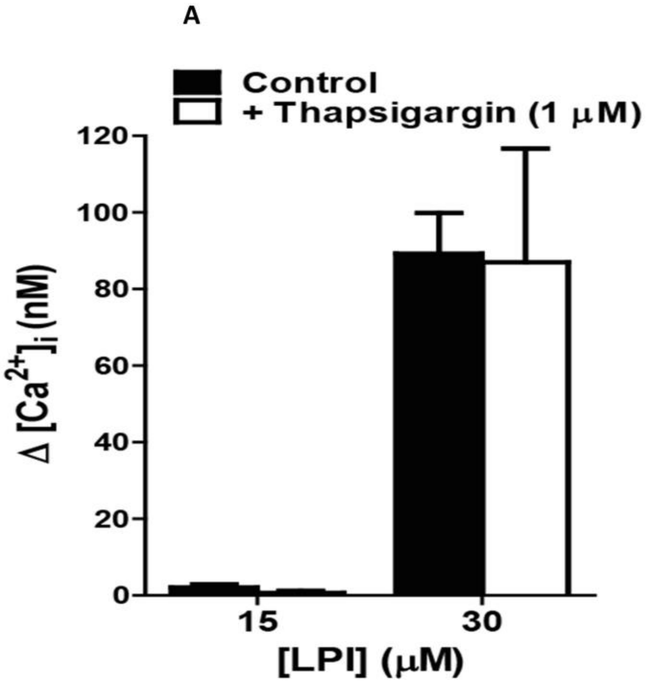

30
25
20
15
10
5
0
■ Control
☐ + Y-27632 (50 μM)
Δ [Ca^{2+}]$_i$ (nM)
5
10
15
20
30
[LPI] (μM)

Figure 7


A
Control
+ Thapsigargin (1 μM)
120
100
80
60
40
20
0
Δ [Ca2+]i (nM)
15
30
[LPI] (μM)

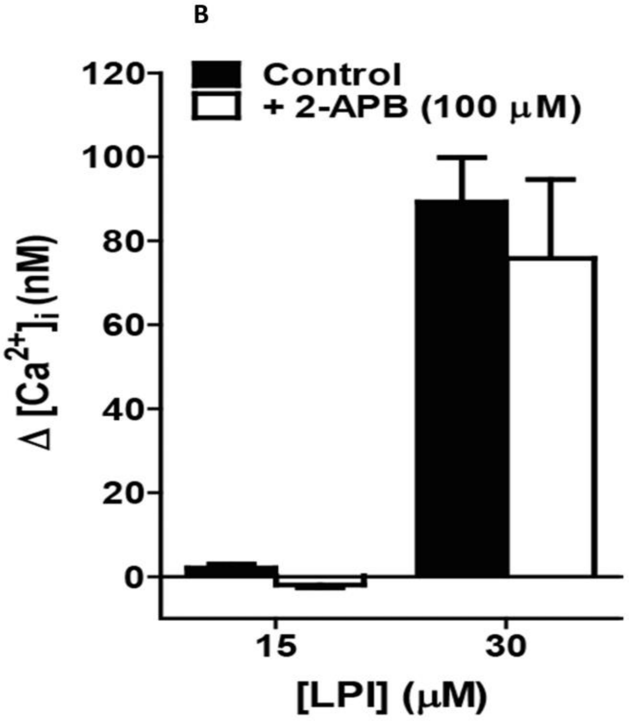
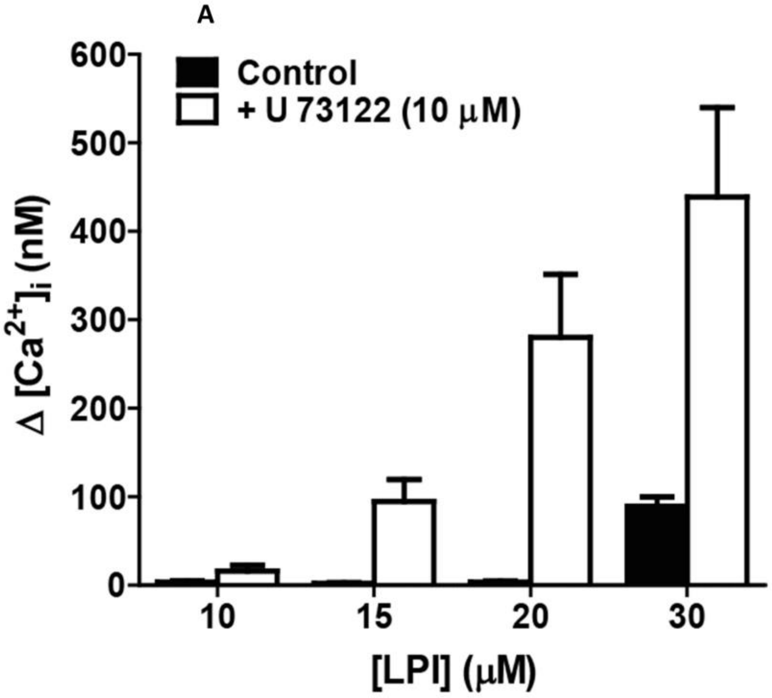

B
Control
+ 2-APB (100 μM)
Δ [Ca2+]i (nM)
120
100
80
60
40
20
0
15
30
[LPI] (μM)

Figure 8


A
Control
+ U 73122 (10 μM)
Δ [Ca2+]i (nM)
600
500
400
300
200
100
0
10
15
20
30
[LPI] (μM)

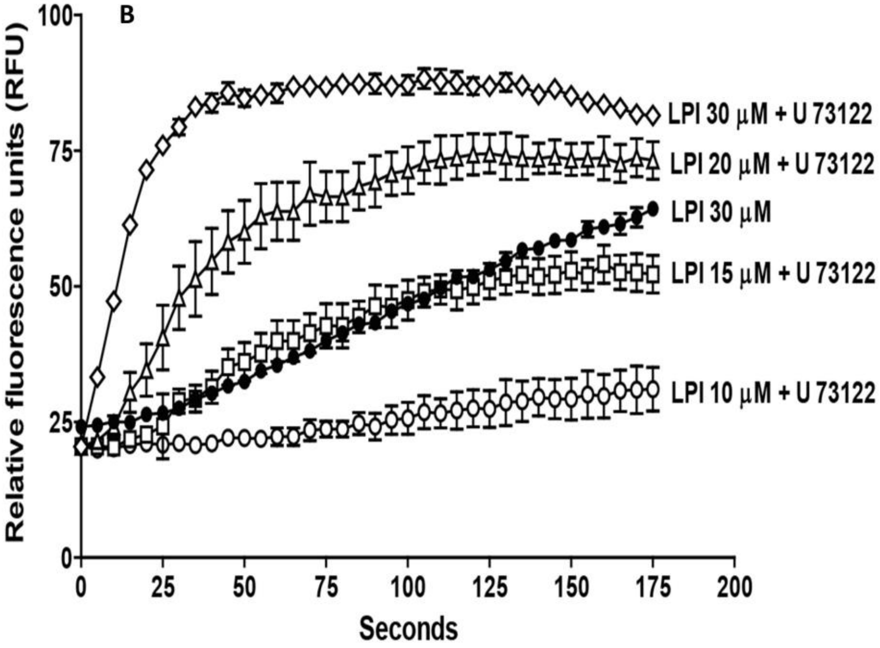
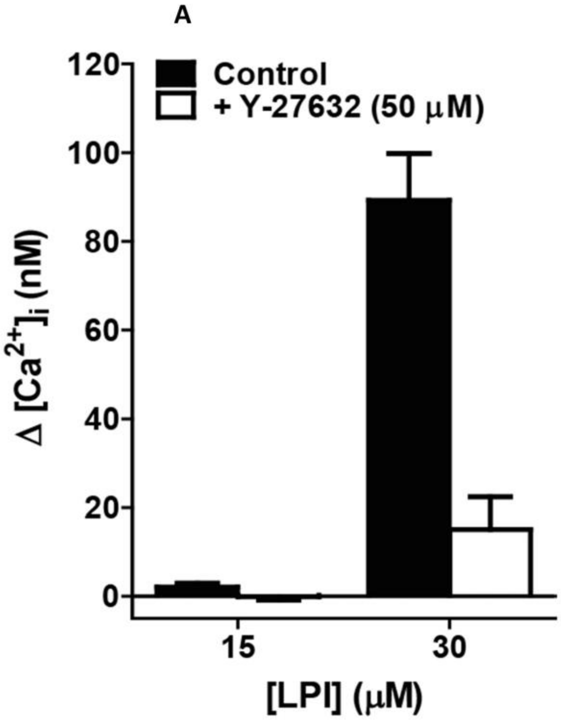
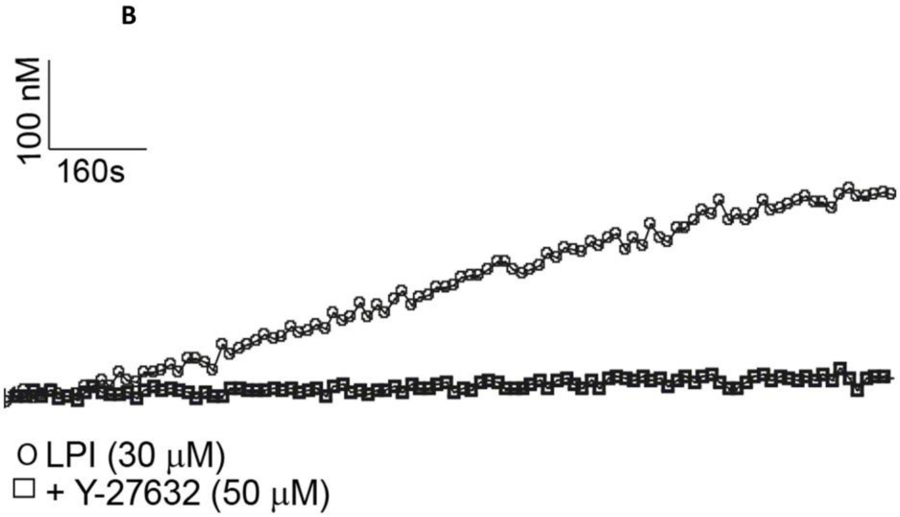

B
Relative fluorescence units (RFU)
100
75
50
25
0
0
25
50
75
100
125
150
175
200
Seconds
LPI 30 μM + U 73122
LPI 20 μM + U 73122
LPI 30 μM
LPI 15 μM + U 73122
LPI 10 μM + U 73122

Figure 9




B
100 nM
160s
○ LPI (30 μM)
□ + Y-27632 (50 μM)