**Effect of Lysophosphatidylglycerol on Intracellular Free Ca2+ Concentration in A10 Vascular Smooth Muscle Cells**

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Effect of Lysophosphatidylglycerol on Intracellular Free $\text{Ca}^{2+}$ Concentration in A10 Vascular Smooth Muscle Cells

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Short Title: Lysophosphatidylglycerol and Smooth Muscle Cells

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Abstract

Although plasma levels of lysophosphatidylglycerol (LPG) are increased in hypertension, its role in the pathogenesis of vascular defects is not clear. In view of the importance of Ca\(^{2+}\)-overload in causing vascular smooth muscle (VSM) dysfunction, the action of LPG on [Ca\(^{2+}\)], in cultured A10 VSM cell line was examined by using Fura 2-AM acetoxymethyl ester technique. LPG was found to induce a concentration-dependent increase in [Ca\(^{2+}\)], in VSM cells. This change was dependent both on the extracellular and intracellular Ca\(^{2+}\) sources as it was reduced by 30% by EGTA, an extracellular Ca\(^{2+}\) chelator, and 70% by thapsigargin, a sarcoplasmic reticulum (SR) Ca\(^{2+}\)-pump inhibitor. However, the increase in [Ca\(^{2+}\)], due to LPG was not altered by caffeine or ryanodine, which affect Ca\(^{2+}\)-release through the ryanodine receptors in the SR. On the other hand, LPG-induced change in [Ca\(^{2+}\)], was suppressed by 2-Nitro-4-carboxyphenyl N,N-diphenylcarbamate, a phospholipase C (PLC) inhibitor, as well as by xestospongion and 2 aminoethoxydiphenyl borate, two inositol trisphosphate (IP3) receptor inhibitors in the SR. These observations support the view that LPG-induced increase in [Ca\(^{2+}\)], in VSM cells is mainly a result of Ca\(^{2+}\)-release from Ca\(^{2+}\) pool in the SR through PLC/IP3-sensitive signal transduction mechanism. Furthermore, it is suggested that the elevated level of LPG may induce intracellular Ca\(^{2+}\)-overload and thus play a critical role in the development of vascular abnormalities.

Keywords: Lysophosphatidylglycerol; vascular smooth muscle cells; intracellular calcium; sarcoplasmic reticulum; vascular abnormalities
Introduction

Intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) plays an important role in the regulation of cardiovascular function, cell growth and apoptosis under both physiological and pathological conditions (Dhalla et al. 1982; Nguyen Dinh Cat and Touyz 2011; Ljubojevic and Bers 2015). A proper [Ca\(^{2+}\)]\(_i\) is required to maintain the normal cardiac contractility and blood pressure as well as other cellular functions in the body (Dhalla et al. 1982). On the other hand, the overload of [Ca\(^{2+}\)]\(_i\) is harmful to cardiovascular health as it is normally seen to occur in cardiac hypertrophy, diastolic heart failure, hypertension and arteriosclerosis (Dhalla et al. 1978; Janczewski and Lakatta 2010; Lipskaia et al. 2013). It is also well known that several vasoactive hormones such as endothelin-1, angiotensin II, vasopressin and serotonin cause increase in the [Ca\(^{2+}\)]\(_i\) in vascular smooth muscle (VSM) cells (Xu et al. 2015). Furthermore, numerous studies have demonstrated that phospholipids and lysophospholipids including phosphatidic acid (PA), lysophosphatidic acid (LPA) and lysophosphatidylglycerol (LPG) may serve as signal molecules in the cells (Dhalla et al. 1997; Mueller et al. 2015). It has been reported that LPG was able to increase insulin release and inhibit the activity of phospholipase D (Metz 1986; Ryu and Palta 2000) and it may serve as a mediator in the cell by binding to the membrane G protein coupled receptors (GPCR) (Park et al. 2007). Intracellular LPG synthesis is mainly dependent on the activity of phospholipase A (PLA) (Makide et al. 2009). Since PLA2 activity is high in several cardiovascular diseases (Rosensor and Hurt-Camejo 2012) and the serum concentration of LPG has been reported to be elevated in the range of 1-2 \(\mu\)M under different pathological conditions associated with high blood pressure as well as in patients with coronary heart disease (Kurano et al. 2015a; Kurano et al. 2015b), we hypothesized that an increase in the level of serum LPG may modify the contraction and relaxation of VSM through its influence on [Ca\(^{2+}\)]\(_i\). It is pointed out
that while extensive work on both PA and LPA has been carried out (Makide et al. 2009; Xu et al. 1996; Xu et al. 2003; Xu et al. 2005; Xu et al. 2008), the biological effects of LPG on cardiovascular system have not been explored. Although LPG has been shown to increase the \([\text{Ca}^{2+}]_i\) in human ovary cancer cells (Park et al. 2007), very little is known regarding its effects on \([\text{Ca}^{2+}]_i\) in VSM cells. Therefore a series experiments were carried out in this study to examine the action of LPG on \([\text{Ca}^{2+}]_i\) in cultured VSM cells by monitoring changes in the intensity of fluorescence of Fura 2 (Xu et al. 1997). Furthermore, mechanisms of the action of LPG in VSM cells were investigated by using the well-established pharmacological approaches (Xu et al. 2005).
Materials and Methods

The A10 VSM cell line was purchased from American Type Culture (Manassas, VA, USA). Dulbecco’s modified eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (ON, Canada). Lysophosphatidylglycerol (LPG), L-α-LPA (c 18:1, [Cis]-9), thapsigargin (TG), 2-Nitro-4-carboxyphenyl N,N-diphenylcarbamate (NCDC), caffeine, 2-aminoethoxydiphenyl borate (2-APB), phorbol 12-myristate 13-acetate (PMA) and ryanodine were purchased from Sigma Chemicals (St. Louis, MO, USA). Xestospongin (Xe C) was obtained from Calbiochem (EMD Biosciences, San Diego, CA, USA). Fura 2-AM was purchased from Molecular Probes (Eugene, OR, USA).

Cell culture:
The A10 VSM cell line was cultured in Petri dish with DMEM containing 10% FBS, 3.7 mg/ml NaHCO₃ and 100 µg/ml gentamicin at 37 °C in the cell culture incubator with 95% air and 5% CO₂ mixture. The culture medium was changed every 2-3 days. The fresh medium was warmed up to 37 °C before use to prevent temperature shock. When the cells became confluent, DMEM containing 2% FBS was used to make the cells quiescent 24 hr prior to the experiments (Xu et al. 2003). Three to six passages of cells were used for the experiments. It is pointed out that A10 VSM cell line has been used previously in our laboratory to investigate intracellular Ca²⁺-signaling pathway (Xu et al. 2005).

[Ca²⁺]ᵢ measurement:
The cultured A10 VSM cells were incubated with 0.25% trypsin-1 mM EDTA for 2-3 min and then the cells were removed from the bottom of the petri dish by the aid of a cell scraper. The
cell suspensions were centrifuged at 240 x g for 5 min at room temperature. The supernatant was discarded and the cells were re-suspended in 10 ml HEPES buffer. In order to load the fluorescence dye, the cells were incubated with 10 µM Fura 2-AM (dissolved in dimethyl sulfoxide with 0.02% pluronic acid) for 30 min at 37°C in HEPES buffer containing in mM: NaCl 145, KCl 4.5, CaCl₂ 1.0, MgSO₄·7H₂O 1.0, HEPES 10, glucose 5, bovine serum albumin 0.1%, KH₂PO₄ 1.0, pH 7.4. To remove extracellular dye, the cells were washed two times with HEPES buffer and the cell number was adjusted to 0.3 × 10⁶ cells/ml. 2 ml of the cell suspension was added to a cuvette with a stirring bar and the base line of fluorescence intensity was recorded. Thereafter 20 µl of LPG was injected into the cuvette and the intensity of fluorescence was recorded for 5 min.

The fluorescence intensity of Fura 2 was determined by a SLM DMX-1100 dual-wavelength spectrofluorometer (SLM Instruments, Inc., Urbana, IL, USA); the ratio (R) of fluorescence signal at the excitation wave length of 340/380 (nm) was calculated automatically at the emission wave length of 510 nm. The R_max and R_min values were determined by the addition of 40 µl Triton X-100 (10%) and 20 µl EGTA (40 mM) to cuvette with 2 ml cell suspension, respectively. The [Ca²⁺]ᵢ was calculated according to the following formula:

\[
[Ca^{2+}]_i = 224 \times \left[ \frac{(R-R_{min})}{(R_{max}-R)} \right] \times \frac{Sf2}{Sb2}
\]

where Sf2 and Sb2 are the fluorescence proportionality coefficients obtained at 380 nm under R_min and R_max conditions, respectively. The method for the measurement of [Ca²⁺]ᵢ is the same as described earlier (Xu et al. 2005). It should be mentioned that concentrations of various pharmacological agents for treatment of VSM cells in this study for studying signal transduction mechanisms are similar to those used in our laboratory previously (Xu et al. 1996; Xu et al. ...
2003; Xu et al. 2005). The pretreatments of cells with all inhibitors, except for EGTA, were carried out for 10 min before exposure to LPG. The responses to LPG were measured at the time of maximal increases in the intensity of fluorescence.

**Statistical Analysis**

The data are presented as mean ± S.E.M. and analyzed statistically by using the Microcal Origin Version 6 (Microcal Software, Inc., MA, USA). The mean values were compared by one-way analysis of variance (ANOVA) and Student’s “t” test. P values < 0.05 indicated significant difference.
Results

LPG-induced a concentration-dependent increase in [Ca\(^{2+}\)]\(_i\)

In order to investigate the effects of LPG on [Ca\(^{2+}\)]\(_i\) in VSM cells, different concentrations of LPG were used. It was found that 1-50 µM of LPG induced a concentration-dependent increase in the intracellular free Ca\(^{2+}\) (Figure 1). In some experiments, LPG at a concentration of 100 µM showed a further increase in [Ca\(^{2+}\)]\(_i\), but the data were not included in Figure 1. It should be mentioned that the addition of LPG caused immediate increase in [Ca\(^{2+}\)]\(_i\), which peaked at about 30 sec and then gradually returned towards the basal value at about 50 sec. Thus all values were measured at maximal response. The basal Ca\(^{2+}\) in the A10 VSM cells in this experiment was found to vary in the range from 70 to 90 nM.

Sources of Ca\(^{2+}\) for the LPG-induced increase in [Ca\(^{2+}\)]\(_i\)

To examine the source of the increase in [Ca\(^{2+}\)]\(_i\)-induced by LPG, the VSM cells were pretreated with ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), an extracellular Ca\(^{2+}\) chelator, or thapsigargin (TG), a Ca\(^{2+}\)-pump ATPase inhibitor, which prevents Ca\(^{2+}\)-uptake in the sarcoplasmic reticulum (SR) (Xu et al. 1996; Xu et al. 2005). In cells pretreated either with EGTA (1 mM) for 30 sec or TG (1 or 10 µM) for 10 min, LPG (20 µM) -induced increase in [Ca\(^{2+}\)]\(_i\) was about 70% or 30% of the control values, respectively (Figure 2). It can also be seen from Figure 2A that the basal [Ca\(^{2+}\)]\(_i\) level was not affected by TG whereas it was reduced by EGTA pretreatment. These observations indicate that both extracellular and intracellular Ca\(^{2+}\) sources contribute to the augmentation of [Ca\(^{2+}\)]\(_i\) induced by LPG.
LPG-induced increase in [Ca$^{2+}$]$_i$ is independent of Ca$^{2+}$-induced Ca$^{2+}$-release channels

Ca$^{2+}$-influx from the extracellular space may trigger Ca$^{2+}$-release from SR Ca$^{2+}$-store through ryanodine receptors, which is named as Ca$^{2+}$-induced Ca$^{2+}$-release channels. To examine if LPG is able to evoke Ca$^{2+}$-release from SR through ryanodine receptors, caffeine (10 mM) and ryanodine (10 or 50 µM) were used in this study. Caffeine keeps the ryanodine receptor open and thus Ca$^{2+}$ storage in this compartment of the SR becomes empty, whereas ryanodine blocks the opening of these channels in the SR (Xu et al. 2005; Xu et al. 2008). It was observed that both caffeine and ryanodine did not affect the amplitude of [Ca$^{2+}$]$_i$ in response to LPG (20 µM) or the basal [Ca$^{2+}$]$_i$ values (Figure 3).

LPG-induced increase in [Ca$^{2+}$]$_i$ is mediated by inositol trisphosphate (IP3) receptors

Besides ryanodine receptors in the SR, some agonists are able to release Ca$^{2+}$ through IP3 receptors in the SR (Xu et al. 2005). To investigate the participation of IP3 in LPG-elicited Ca$^{2+}$ increase, the A10 VSM cells were incubated with NCDC (10 or 50 µM) for 10 min before the addition of LPG. NCDC is a phospholipase C (PLC) inhibitor, which prevents the formation of IP3 in the cells (Xu et al. 1997). As shown in Figure 4A, NCDC at 50 µM reduced the basal level of [Ca$^{2+}$]$_i$ whereas NCDC at both 10 µM and 50 µM concentrations showed inhibitory effect on the LPG-induced change in [Ca$^{2+}$]$_i$ (Figure 4B). To determine whether inositol trisphosphate receptor (InsP3R) contributes to the LPG-induced increase in [Ca$^{2+}$]$_i$, two InsP3R receptors blockers, Xe C (10 µM) and 2-APB 20 µM (Xu et al. 2005), were used in the same way as NCDC; both these insP3R antagonists had no effect on basal [Ca$^{2+}$]$_i$ (Figure 4C) but inhibited the LPG (20 µM)-induced response by about 50-60% (Figure 4D).
LPG-induced increase in [Ca^{2+}]_i is not affected by protein kinase C (PKC)

Various studies have reported that PKC activation modifies the intracellular Ca^{2+} homeostasis (Paffett et al. 2010). The role of PKC in LPG-induced changes in [Ca^{2+}]_i was studied by the incubation of the A10 VSM cells with two doses of PMA (0.1µM or 10 µM), a PKC activator, for 10 min. The pretreatment with PMA had no significant effect on the basal [Ca^{2+}]_i level or LPG (20 µM)-evoked increase in [Ca^{2+}]_i (Figure 5A and B).

LPG attenuates the LPA-induced increase in [Ca^{2+}]_i

LPA and LPG have similar chemical structure and both belong to the lysophospholipids family. The effects of LPA on [Ca^{2+}]_i in VSM cells are well documented in the literature (Xu et al. 2005). Previous studies have demonstrated that exposure of the cells to LPG blunted the LPA-elicited changes in [Ca^{2+}]_i in human Jurkat T cell line (Xu et al. 1995). To gain some information if LPG and LPA caused Ca^{2+}-release from the same Ca^{2+} store, the A10 VSM cells were incubated with 20 µM of LPG for 10 min prior to challenging with LPA (10 µM). As shown in the Figure 6A, pretreatment of A10 VSM cells by LPG increased basal [Ca^{2+}]_i significantly (P<0.05); however, the LPA-induced elevation of [Ca^{2+}]_i was inhibited about 60% by the pretreatment of the cells with LPG (Figure 6B).
Discussion

In this study, LPG was found to induce a concentration-dependent increase in \([\text{Ca}^{2+}]_i\) in A10 VSM cells; LPG at the concentration of 1 µM was able to increase \([\text{Ca}^{2+}]_i\) significantly. Since the serum concentration of LPG has been reported to vary in the range of 1-2 µM under pathological conditions associated with high blood pressure and cardiac dysfunction (Kurano et al. 2015a, Kurano et al. 2015b), it is likely that LPG may play a role in eliciting cardiovascular abnormalities as a consequence of its action on \([\text{Ca}^{2+}]_i\). Particularly, in view of the involvement of \([\text{Ca}^{2+}]_i\) in VSM cell proliferation and stiffness of the blood vessels (Xu et al. 1996), the sustained elevation of serum LPG level can be seen to contribute to the development of atherosclerosis, hypertension and other vascular defects.

It has been demonstrated that the increase in \([\text{Ca}^{2+}]_i\) may result from either \(\text{Ca}^{2+}\)-influx from the extracellular space or release from the SR \(\text{Ca}^{2+}\)-stores in VSM cells (Xu et al. 2005). Since incubation of the cells with EGTA attenuated the LPG-induced increase in \([\text{Ca}^{2+}]_i\) by about 30%, it appears that extracellular \(\text{Ca}^{2+}\) may partially contribute to the action of LPG. The influx of the \(\text{Ca}^{2+}\) from extracellular space may trigger \(\text{Ca}^{2+}\)-release from SR through ryanodine receptors, referred to as \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\)-release. Ryanodine and caffeine are two agents, which are commonly used for this kind study (Xu et al. 1996; Xu et al. 2005; Xu et al. 2008). Our study has demonstrated that neither ryanodine nor caffeine affected the action of LPG and thus it is suggested that LPG did not cause \(\text{Ca}^{2+}\)-release through ryanodine receptors in the SR. In contrast, it was reported that the LPA-elicited increase in \([\text{Ca}^{2+}]_i\) was inhibited by 30-50% by these two agents in A10 VSM cells (Xu et al. 2005). Such a difference in the LPA and LPG responses to ryanodine and caffeine seems to indicate that the mechanisms for these two agonists...
to increase the \([\text{Ca}^{2+}]_i\) in VSM cells may be different from each other. Previously, we observed that the LPA which acts on the membrane receptor, \(\text{Ca}^{2+}\) channels and \(\text{Ca}^{2+}\) storage in the SR induced increase in \([\text{Ca}^{2+}]_i\) in VSM cells was attenuated by incubation of the cells with PMA (Xu et al. 2005). Since the action of LPG on \([\text{Ca}^{2+}]_i\) was not affected by PMA, this observation also suggests difference between the mechanisms of LPG and LPA.

Earlier studies have shown that the binding of LPA to GPCR in the membrane would stimulate PLC and release IP3 from the membrane to the cytosol. The binding of IP3 to its receptor may cause \(\text{Ca}^{2+}\)-release from IP3-sensitive pool in the SR (Xu et al. 2005; Choi et al. 2013) and thus the effect of LPA on the augmentation of \([\text{Ca}^{2+}]_i\) was suggested to be partially mediated through this pathway. Xu et al (1995) have observed that the LPG-induced change in \([\text{Ca}^{2+}]_i\) was inhibited by NCDC, a PLC inhibitor, in Jurkat T cell line. Park et al (2007) have also reported similar findings in human ovarian cancer cells. Our observation with NCDC in the VSM cells is in good agreement with these reports (Park et al. 2007; Xu et al. 1995). In addition, we have used two more IP3 receptor blockers, Xe C and 2-APB, in the present study and have found that both these IP3 receptor inhibitors suppressed the LPG-induced increase in \([\text{Ca}^{2+}]_i\) by 50 to 60%. These findings further confirm the participation of IP3 pathway in LPG-induced \(\text{Ca}^{2+}\)-release from the SR.

Xu et al. (1995) have reported that LPG is a competitive inhibitor of LPA receptor because it suppressed the LPA-induced elevation in \([\text{Ca}^{2+}]_i\) in Jurkat T cell line. We observed that incubation of VSM cells with LPG partially inhibited the LPA-induced response. It is possible that LPG may cause \(\text{Ca}^{2+}\)-release from the SR \(\text{Ca}^{2+}\)-stores by two pathways: one is the same as
that for LPA and the other route is different from that for LPA; this may explain the partial inhibition of LPA effect by LPG. On the other hand, Park et al. (2007) have suggested that the LPG receptor as well as LPA receptors (LPA1, LPA2 and LPA3) may be different from each other since LPG failed to produce any response in the exogenously transfected LPA receptors in the HepG cells. However, at present no direct evidence of LPG receptors is available in the literature (Makide et al. 2009). Since several investigators have reported the interaction of LPA, LPG and prostaglandins (Sparkes et al. 2010; Yoshimoto et al. 2016; Boruszewska et al. 2017), the involvement of prostaglandins in mediating the actions of LPG cannot be ruled out.

In conclusion, LPG was found to cause a concentration-dependent increase in [Ca$^{2+}$]$_i$ in cultured A10 VSM cell line, which was dependent on both extracellular and intracellular Ca$^{2+}$ sources. The release of Ca$^{2+}$ from IP3-sensitive SR Ca$^{2+}$ pool seems to contribute mainly to the increase in [Ca$^{2+}$]$_i$-induced by LPG. It should be pointed out that there are several limitations in the present study and thus some caution should be exercised when interpreting the data. The in vitro experiments with cultured A10 VSM cells may be different from human VSM cells as well as in their response in vivo. Furthermore, the IP3 concentration in these cells with or without exposure to LPG was not measured in this study while alterations in [Ca$^{2+}$]$_i$ due to IP3-sensitive SR Ca$^{2+}$ pool were based on the use of some of its non-specific inhibitors. It is emphasized that extensive experiments in the presence of different eicosanoid inhibitors remain to be carried out to determine the role of prostaglandins in LPG-induced increase in [Ca$^{2+}$]$_i$ in VSM cells. Since we did not measure the LPG-induced changes in the contraction of vascular cells used in this study, some caution should also be exercised in interpreting the relationship between changes in [Ca$^{2+}$]$_i$ and vascular contractile activity. Nonetheless, the results presented in this study provide...
some evidence that LPG may play a role in the pathogenesis of vascular diseases associated with increased level of \([\text{Ca}^{2+}]_i\).

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**Conflict of Interest**

None
References


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Figure legends

Figure 1. Responses of intracellular free calcium concentration ([Ca$^{2+}$]$_i$) to different concentrations of lysophosphatidylglycerol (LPG) in A10 vascular smooth muscle (VSM) cells. Each bar graph represents the value of mean ± S.E.M. of six preparations in each group.

Figure 2. Effects of EGTA and thapsigargin (TG) on lysophosphatidylglycerol (LPG)-induced increases in intracellular free calcium concentration ([Ca$^{2+}$]$_i$) in A10 vascular smooth muscle (VSM) cells. EGTA (1 mM) was injected to the curette 30 sec prior to the addition of LPG (20 µM), whereas two concentrations of TG (TG1, 1 µM; TG10,10 µM) were incubated with the cells for 10 min before challenging the cells with LPG. The values are expressed as mean ± S.E.M. of six determinations in each group. *P < 0.05 vs control (-).

Figure 3. Effects of caffeine (CAF) and ryanodine (RYA) on lysophosphatidylglycerol (LPG)-induced increase in intracellular free calcium concentration ([Ca$^{2+}$]$_i$) in A10 vascular smooth muscle (VSM) cells. CAF (10 mM) and two concentrations of RYA (RYA10, 1 µM; RYA50, 50 µM) were incubated with the cells for 10 min before challenging the cells with LPG (20 µM). The values are expressed as mean ± S.E.M. of six preparations in each group.

Figure 4. Effects of NCDC, Xe C and 2-APB on lysophosphatidylglycerol (LPG)-induced increase in intracellular free calcium concentration ([Ca$^{2+}$]$_i$) in A10 vascular smooth muscle (VSM) cells. Two concentrations of NCDC (NC10, 10 µM; NC50, 50 µM.), Xe C (XEC 10 µM) and 2 APB (2APB, 20 µM) were incubated with the cells for 10 min before challenging the VSM cells with LPG (20 µM). The values are expressed as mean ± S.E.M. of four preparations.
in each group. *-P < 0.05 vs control (-).

Figure 5. Effects of protein kinase C stimulator PMA on lysophosphatidylglycerol (LPG)-induced increase in intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) in A10 vascular smooth muscle (VSM) cells. Two concentrations of PMA (PMA0.1, 0.1 μM; PMA10, 10 μM.) were incubated with the cells for 10 min before adding LPG (20 μM). The values are expressed as mean ± S.E.M. of four preparations in each group.

Figure 6. Effects of lysophosphatidylglycerol (LPG) on lysophosphatidic acid (LPA)-induced increases in intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) in A10 vascular smooth muscle (VSM) cells. 20 μM of LPG was incubated with the cells for 10 min before adding LPA (10 μM). A: the effects of LPG on the basal [Ca\(^{2+}\)]\(_i\) after 10 min incubation; B: the increase in [Ca\(^{2+}\)]\(_i\) in the presence or absence of LPG (20 μM). The values are expressed as mean ± S.E.M. of six determinations in each group. *-P < 0.05 vs control (-).