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Mechanisms of the lysophosphatidic acid-induced increase in $[Ca^{2+}]_i$ in skeletal muscle cells

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Abstract

Although lysophosphatidic acid (LPA) is known to increase intracellularfree calcium concentration ($[Ca^{2+}]_i$) in different cell types, the effect of LPA on the skeletal muscle cells is not known. The present study was therefore undertaken to examine the effect of LPA on the $[Ca^{2+}]_i$ in C2C12 cells. LPA induced a concentration and time dependent increase in $[Ca^{2+}]_i$, which was inhibited by VPC12249, VPC 32183 and dioctanoyl glycerol pyrophosphate, LPA1/3 receptor antagonists. Pertussis toxin, a G^i protein inhibitor, also inhibited the LPA-induced increase in $[Ca^{2+}]_i$. Inhibition of tyrosine kinase activities with tyrphostin A9 and genistein also prevented the increase in $[Ca^{2+}]_i$ due to LPA. Likewise, wortmannin and LY 294002, phosphatidylinositol 3-kinase (PI3-K) inhibitors, inhibited $[Ca^{2+}]_i$ response to LPA. The LPA effect was also attenuated by ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), an extracellular Ca^{2+} chelator, Ni^{2+} and KB-R7943, inhibitors of the Na^+-Ca^{2+} exchanger; the receptor operated Ca^{2+} channel (ROC) blockers, 2-aminoethoxydiphenyl borate and SK&F 96365. However, the L-type Ca^{2+} channel blockers, verapamil and diltiazem; the store operated Ca^{2+} channel blockers, La^{3+} and Gd^{3+}; a sarcoplasmic reticulum calcium pump inhibitor, thapsigargin; an inositol trisphosphate receptor antagonist, xestospongin and a phospholipase C inhibitor, U73122, did not prevent the increase $[Ca^{2+}]_i$ due to LPA. Our data suggest that the LPA-induced increase in $[Ca^{2+}]_i$ might occur through G^i-protein coupled $LPA_{1/3}$ receptors that may be linked to tyrosine kinase and PI3-K, and may also involve the Na^+-Ca^{2+} exchanger as well as the ROC. In addition, LPA stimulated C2C12 cell proliferation *via* PI3-K. Thus, LPA may be an important phospholipid in the regulation of $[Ca^{2+}]_i$ and growth of skeletal muscle cells.

Keywords: lysophosphatidic acid • skeletal muscle cell • intracellular free calcium • receptor operated Ca^{2+}-channels • sarcolemmal Na^+-Ca^{2+} exchanger

Introduction

Lysophosphatidic acid (LPA), a water-soluble bioactive lysophospholipid, is present in both the serum and the cytosol compartment of the cell. The cytosolic LPA is formed as a metabolite of phosphatidic acid (PA) by the action of phospholipase A_2 [1], whereas, the serum LPA is mainly formed due to hydrolysis of lysophosphatidylcholine by phospholipase D (PLD) and is released into the serum from platelets and fibroblasts [2]. The concentration of LPA varies from 1 to 5 μM under physiological conditions and can reach up to 20 μM in response to injury and inflammation [3].

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G-protein coupled receptors (GPCRs) are known to regulate a diverse range of physiological processes in response to a variety of agonists [4], however, the function of the majority of GPCRs expressed in skeletal muscle remains largely unknown [5]. The biological effects of LPA are considered to be mediated by at least five GPCRs, designated as LPA_{1-5} [6–8]. The downstream effectors appear to be even more diverse and include activation of phospholipase C (PLC), tyrosine kinases and phosphatidylinositol 3-kinase (PI3-K) [9–13]. LPA receptors are known to be expressed in skeletal muscle cells and are considered to participate in the mitogenic effects and apoptosis [6, 14, 15]. In addition, LPA contained in serum has been reported to stimulate the proliferation and inhibit the differentiation of myoblast cells [15]; these actions of LPA were blocked by pertussis toxin (PTX), suggesting the involvement of G_i proteins in the LPA response [16]. Exogenous LPA can increase $[Ca^{2+}]_i$ in different cell types [17–20] and has been reported to activate PI3-K in cardiomyocytes [21]. In addition, PI3-K has been demonstrated to regulate Ca^{2+} in T cells [17], suggesting a possibility that PI3-K might also be involved in mediating the LPA response in skeletal muscle.

In view of the critical importance of $[Ca^{2+}]_i$ in the regulation of skeletal muscle function [22–25], the present study was therefore undertaken to examine if LPA can induce an increase in $[Ca^{2+}]_i$, to identify the sources of Ca^{2+} mobilization as well as to determine the signal transduction mechanisms that might be involved in mediating the LPA response in skeletal muscle C2C12 cells. Our results show for the first time that LPA can induce an increase in cytosolic and nuclear $[Ca^{2+}]$ as well as DNA synthesis in skeletal muscle cells and may therefore play an important role in the regulation of $[Ca^{2+}]_i$ and growth of skeletal muscle cells.

Materials and methods

Cell culture

C2C12 skeletal muscle cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, ON, Canada) containing 10 % foetal bovine serum (FBS, Invitrogen, ON, Canada.) at 37°C with 95 % air and 5 % CO_2. When the cells reached to 80 % of the confluence, they were incubated in DMEM containing 2 % FBS for a further 24 hrs prior to the experiments [26, 27].

Measurement of $[Ca^{2+}]_i$ in C2C12 cell suspension

The LPA (L-α-LPA, oleoyl (C18:1, [Cis]-9), Sigma-Aldrich, MO, USA)-induced changes in $[Ca^{2+}]_i$ were measured according to methods previously described by Xu *et al.* [26]. Briefly, C2C12 cells were washed twice with serum free DMEM and then harvested with 0.25 % trypsin-1 mM ethylenediaminetetraacetic acid (EDTA). The cells were centrifuged at 240 g for 5 min. at room temperature. The supernatant was removed and the cells were washed two times in *N*-2-hydroxyethylpiperazine-N´-2-ethane sulfonic acid (HEPES) buffer containing NaCl 145 mM, KCl 4.5 mM, $CaCl_2$ 1.0 mM, $MgSO_4.7H_2O$ 1.0 mM, HEPES 10 mM, glucose 5 mM, KH_2PO_4 1.0 mM, bovine serum albumin (BSA) 0.1 % (pH 7.4) and were incubated for 40 min at 37°C with 10 μM fura-2/AM (Molecular Probes, OR, USA) in HEPES buffer. The cells were then washed twice with HEPES buffer to remove the extracellular dye and their number was adjusted to approximately 0.3 x 10^6 cells/ml. The cell suspension in the cuvette was stirred at the speed of 500 rpm. The fluorescence intensity of fura-2 was monitored by a SLM DMX-1100 dual-wavelength spectroflurometer; the ratio (R) of fluorescence signal at 340/380 (nM) was calculated automatically. The R_{max} and R_{min} values were determined by the addition of 40 μl Triton X-100 (10%) and 20 μl ethylene glycol-bis (β-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA, 400 mM), respectively. The $[Ca^{2+}]_i$ was calculated according to the following formula: $[Ca^{2+}]_i = 224 \times [(R-R_{min})/(R_{max}-R)] \times Sf_2/Sb_2$, where Sf_2 and Sb_2 are the fluorescence proportionality coefficients obtained at 380 nm under Rmin and Rmax conditions, respectively [26, 27]. The pre-treatment of cells with the various pharmacological agents was performed by incubating cells in the buffer containing desired concentration of the different agents for 10 min at room temperature, except for the pre-treatment with $LPA_{1/3}$ receptor antagonists, which was for 10 sec, prior to the measurement of fluorescence in response to LPA. In the experiments requiring pre-treatment with inhibitors, similar volume of vehicle, dimethyl sulfoxide (DMSO) or water, was added to the control group. The increase in $[Ca^{2+}]_i$ at the peak transient was calculated as the net increase above the basal value in each experiment in response to LPA in the absence and presence of different interventions.

Determination of $[Ca^{2+}]_i$ in single C2C12 cell

The measurement of $[Ca^{2+}]_i$ in single cell was performed in confocal microscope (Nikon TE2000). The cells were cultured on the Glass Bottom Culture Dishes (MatTek, Ashland, MA, USA) for 6 days, and then the cells were loaded with 10 μM of fluo-3/AM in HEPES buffer for 30 min. After washing three times with HEPES buffer, the cells was

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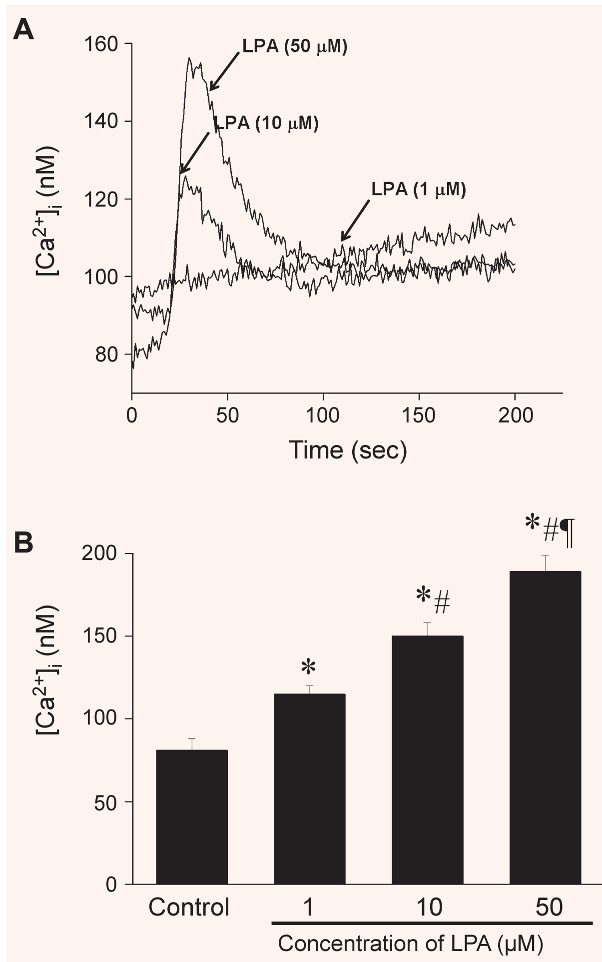

Fig. 1 Concentration-dependent effect of lysophosphatidic acid on intracellular calcium concentration in C2C12 skeletal muscle cells. (**A**): Real time recording of $[Ca^{2+}]_i$ in C2C12 cells. **B**: Quantified data of the LPA-induced increases in $[Ca^{2+}]_i$ in C2C12 cells. Values are means ± S.E.M. of six different experiments. Cultured cells were trypsinized using 2 ml 0.25 % trypsin-1 mM ethylenediaminetetraacetic acid (EDTA) and washed twice with HEPES buffer. The cells were then incubated with 10 μM fura-2/AM in HEPES buffer for 40 min and then washed twice with HEPES buffer to remove any extracellular dye. The fluorescence was monitored by a SLM DMX-1100 dual wavelength spectrofluorometer. *P<0.05 *versus* control value. #P < 0.05 *versus* 1 μM LPA. ¶P < 0.05 *versus* 10 μM LPA. LPA: lysophosphatidic acid, $[Ca^{2+}]_i$: intracellular calcium concentration.

scanned in Time Series and the fluo-3 is excited by 488 nm argon laser. The $[Ca^{2+}]_i$ was expressed by the intensity of fluorescence of fluo-3 [28]. The confocal data were analysed by EZ C1 program and Simple PCI (Version 5.1).

Assessment of DNA synthesis in C2C12 cells

The LPA-induced DNA synthesis in C2C12 cells was determined by measuring [^{3}H]-thymidine (Amersham, ON, Canada) incorporation by pulse labelling as described previously [27]. Cells in 12-well plates were serum starved for 20 hrs for synchronization, washed twice with serum-free medium and incubate with 0.5 ml serum-free medium. LPA was added in the presence of 100 nM wortmannin (Calbiochem, CA, USA) or 2 μl DMSO to control cells. After incubation for 4 hrs, 1 μCi [^{3}H]-thymidine was added. The reaction was stopped after 22 hrs by removing the medium on ice. The cells were washed three times with 1 ml HEPES buffer. These cells were then incubated for 1 hr in ice cold 5 % trichloroacetic acid (TCA) on the ice, washed two more times with 0.5 ml HEPES buffer, and incubated with 0.2 ml NaOH (0.5 N) for a period of 1 hr. The aliquots were transferred to scintillation vial. The radioactivity was counted in a Beckman LS 6500 scintillation counter after the addition of 10 ml scintillant (CytoScint, ICN, CA, USA).

Statistical analysis

The data are expressed as mean ± S.E.M. Statistical analysis was performed using the Microcal Origin Version 6 (Microcal Software Inc., MA, USA). The differences between two groups were evaluated by Student's t-test. The data from more than two groups were evaluated by One-Way ANOVA followed by Student's t-test for the comparison between control and treated group. The value of P less than 5 % (P< 0.05) was considered to be statistically significant.

Results

Concentration-dependent effect of LPA on $[Ca^{2+}]_i$ in C2C12 cells

We have previously reported that LPA increases $[Ca^{2+}]_i$ in A10 vascular smooth muscle cells (VSMCs) [26, 27]. Likewise, LPA (1–50 μM) induced a significant concentration-dependent increase in $[Ca^{2+}]_i$, with the maximum response at 30 sec that declined back to basal levels at 2 min (Fig. 1A). A maximum response was achieved at 50 μM LPA (Fig. 1A and B). A concentration of 10 μM LPA was subsequently used for the investigation of the mechanisms of the LPA-induced increase in $[Ca^{2+}]_i$. This was also based on other studies that have employed

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this concentration of LPA [21, 26, 27]. It should be noted 20 μl water served as a control and exerted no significant effect on basal $[Ca^{2+}]_i$ (data not shown).

Changes in $[Ca^{2+}]_i$ in C2C12 cells in response to LPA in the presence and absence of LPA receptor antagonists and G_i protein inhibition

In order to determine if the LPA-induced increase in $[Ca^{2+}]_i$ was a LPA-receptor mediated response, C2C12 cells were pre-treated with $LPA_{1/3}$ receptor antagonists, dioctanoyl glycerol pyrophosphate (DGPP 8:0, 10 μM), VPC12249 (10 μM) or VPC 32183 (10 μM) [29–31] (Avanti Polar Lipids, Inc, Al, USA) for 10 sec before the addition of LPA. The pre-treatment with these receptor antagonists had no significant effect on the basal $[Ca^{2+}]_i$ (data not shown). However, these receptor blockers significantly inhibited the LPA-induced increase in $[Ca^{2+}]_i$ (Fig. 2). Furthermore, to understand the LPA receptor signalling mechanisms, C2C12 cells were also pre-treated with a G_i protein inhibitor, pertussis toxin (PTX, 100 ng/ml, Sigma-Aldrich, MO, USA) [32] for 10 min. This time of pre-treatment with PTX is based on previous experience [21]. It can be seen in Figure 2 that the LPA-induced increase in $[Ca^{2+}]_i$ in C2C12 cells was significantly inhibited.

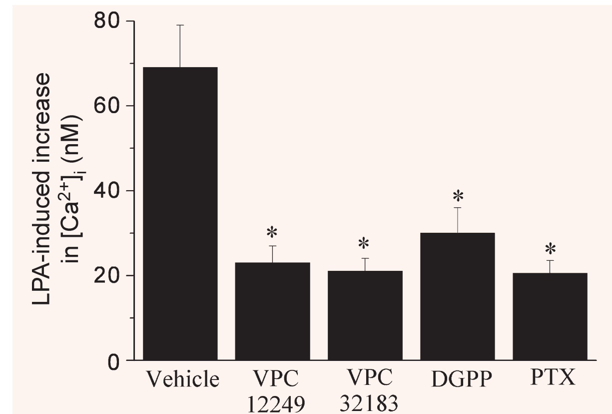

Fig. 2 Effect of lysophosphatidic acid receptor antagonists and G_i protein inhibitor on LPA-induced changes in intracellular Ca^{2+} concentration in C2C12 cells. C2C12 cells were pre-treated with 10 μM of the LPA receptor antagonists, VPC 12249, VPC 32183 and DGPP (8.0), for 10 sec or with pertussis toxin (PTX, 100 ng/ml), a G_i protein inhibitor, for 10 min prior to the addition of LPA (10 μM). The $[Ca^{2+}]_i$ was measured as described in the Materials and methods. Values are means ± S.E.M. of six different experiments. $^*P < 0.05$ *versus* vehicle control value. DGPP: dioctanoyl glycerol pyrophosphate; LPA : lysophosphatidic acid, $[Ca^{2+}]_i$: intracellular calcium concentration.

Role of tyrosine kinase and phosphatidylinositol 3-kinases inhibitors in the LPA-induced increase in $[Ca^{2+}]_i$ in C2C12 cells

To determine the role of tyrosine kinase on LPA-induced increase in $[Ca^{2+}]_i$ in C2C12 cells, genistein (5 μM, Sigma-Aldrich, MO, USA) and tyrphostin A9 (A9, 5 μM, Sigma-Aldrich, MO, USA), tyrosine kinase inhibitors [33], were added 10 min prior to the addition of LPA. A significant attenuation of the LPA-induced Ca^{2+} response was seen by both inhibitors (Fig. 3A). The role of PI3-K in the LPA-induced increase in $[Ca^{2+}]_i$ was examined by pre-treating C2C12 cells with the PI3-K inhibitors [17], wortmannin (100 nM) and LY 294002 (10 μM) for 10 min prior to addition of LPA. It can be seen in Figure 3B that both wortmannin and LY 294002 significantly attenuated the Ca^{2+} response to LPA by 57 % and 63 %, respectively. It should be noted that the pre-treatment the cells with genistein significantly reduced the basal $[Ca^{2+}]_i$ (vehicle control: 95±3; genistein: 63±3, P<0.05). Furthermore, pre-treatment with other agents had no significant effect on the basal $[Ca^{2+}]_i$ (vehicle control: 90±10 nM; A9: 85±11 nM; wortmannin: 80±6 nM; LY 294002 85±7 nM).

Ca^{2+} influx due to LPA

In C2C12 cells, EGTA (1 mM) reduced both the basal as well as the peak $[Ca^{2+}]_i$ response evoked by LPA, whereas verapamil and diltiazem had no significant effect on the basal and LPA-induced increase in $[Ca^{2+}]_i$ (Table 1), Similarly, both Gd^{3+} and La^{3+}, blockers of store operated Ca^{2+} channels (SOCs), did not exert any significant effect on LPA-evoked increase in $[Ca^{2+}]_i$ (Table 1). To test whether receptor operated Ca^{2+} channels (ROCs) play a role in LPA-induced changes in $[Ca^{2+}]_i$ in C2C12 cells, cells were pre-treated with SK&F 96365

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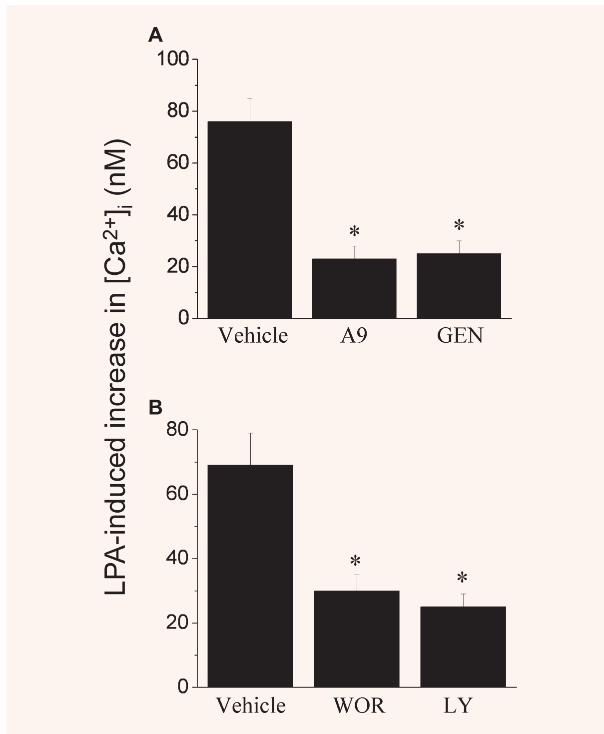

Fig. 3 Effect of tyrosine kinase inhibitors (A) and phosphatidylinositol kinase 3 inhibitors (B) on LPA-induced changes in intracellular calcium concentration in C2C12 cells. C2C12 cells were pre-treated with 5 μM of the tyrosine kinase inhibitors, tyrphostin A9 (A9) and genistein (GEN), and 100 nM or 10 μM of the phosphatidylinositol 3-kinase inhibitors, wortmannin (WOR) and LY 294002 (LY), respectively, for 10 min prior to the addition of LPA (10 μM). The $[Ca^{2+}]_i$ was measured as described in the Materials and methods. Values are means ± S.E.M. of six different experiments. $^*P<0.05$ *versus* vehicle control value. LPA: lysophosphatidic acid, $[Ca^{2+}]_i$: intracellular calcium concentration.

(10 μM, Biomol Research Laboratory, PA, USA), an inhibitor of ROCs [34] and with the transient receptor potential (TRP) C7 cation channel blocker, 2-aminoethoxydiphenyl borate (2-APB, 100 μM, Sigma-Aldrich, MO, USA) [35] for 10 min. Both these agents significantly attenuated the LPA-induced increase in $[Ca^{2+}]_i$ by 50–60 % (Fig. 4A). The cells pre-treated with SK&F 96365 and 2-APB did not alter the basal $[Ca^{2+}]_i$ (vehicle control 81±8; SK&F 96365 81±11; 2-APB 100±12 nM). Pre-treatment with the Na^+–Ca^{2+} exchanger inhibitors, Ni (1 mM) and KB-R7943 (30 μM, Tocris Biosciences,

Table 1 Effect of different agents on Ca^{2+} influx into C2C12 skeletal muscle cells in response to exogenous LPA

Group	Basal $[Ca^{2+}]_i$ (nM)	LPA-induced increase in $[Ca^{2+}]_i$ above the basal (nM)
Water (10 μl)	85 ± 15	76
EGTA (1 mM)	58 ± 6*	30 ± 5*
Verapamil (10 μM)	96 ± 6	72 ± 15
Diltiazem (10 μM)	95 ± 10	72 ± 8
Gd^{3+}(1 μM)	94 ± 21	64 ± 6
La^{3+} (10 μM)	98 ± 19	68 ± 10

Values are means ± S.E.M. of six different measurements. C2C12 cells were pre-treated with EGTA for 30 sec, whereas pre-treatment with the other agents was for 10 min prior to addition of LPA (10 mM). The $[Ca^{2+}]_i$ was measured as described in the Materials and methods. $^*P<0.05$ *versus* vehicle control (water) group.

MO, USA) [26], resulted in a significant inhibition (40–55%) of the LPA-induced increase in $[Ca^{2+}]_i$ in C2C12 cells (Fig. 4B). The pre-treatment of cells with Ni and KB-R7943 alone exerted no significant action on the basal $[Ca^{2+}]_i$ (vehicle control: 83±11 nM; Ni: 81±11 nM; KB-R7943: 91±12 nM).

Sarcoplasmic reticulum (SR) Ca^{2+} release in response to LPA

To determine the intracellular source for the LPA-induced increase in $[Ca^{2+}]_i$, the following series of experiments were conducted. Pre-incubation of C2C12 cells with U73122 (1 μM, Sigma-Aldrich, MO, USA), a PLC inhibitor; xestospongin (XEC, 10 μM, Calbiochem, CA, USA), a membrane permeable inositol trisphosphate (IP_3) receptor blocker and thapsigargin (5 μM, Sigma-Aldrich, MO, USA), which depletes IP_3 sensitive SR Ca^{2+} store [6], did not produce any significant inhibition on the LPA-evoked increase in $[Ca^{2+}]_i$ (Table 2). The effect of 10 mM caffeine (final concentration) was also examined. As shown in Table 2, caffeine alone caused a significant increase in $[Ca^{2+}]_i$, but it did not produce any effect on the LPA-induced increase in $[Ca^{2+}]_i$.

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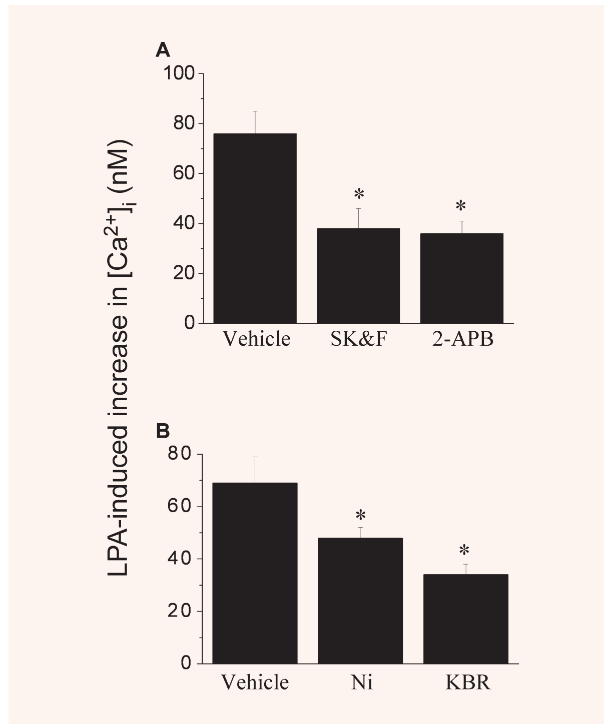

Fig. 4 Effect of receptor operated Ca^{2+} channels (A) and Na^+–Ca^{2+} exchanger inhibitors (B) on lysophosphatidic acid-induced increase in intracellular calcium concentration in C2C12 cells. C2C12 cells were pre-treated with 2-aminoethoxydiphenyl borate (2-APB, 100 μM), SK&F 96365 (10 μM), Ni^{2+} (1 mM) or KB-R7943 (KBR, 30 μM) for 10 min prior to the addition of LPA (10 μM). The $[Ca^{2+}]_i$ was measured as described in the Materials and methods. Values are means ± S.E.M. of six different experiments. *P<0.05 *versus* vehicle control value. LPA: lysophosphatidic acid, $[Ca^{2+}]_i$: intracellular calcium concentration.

Although pre-treatment (30 sec) of the C2C12 cells with 30 mM KCl alone induced a small, but significant increase in the basal $[Ca^{2+}]_i$, however, the LPA-induced increase of $[Ca^{2+}]_i$ that was inhibited by approximately 50 % (Table 2).

Localization of the increase in $[Ca^{2+}]_i$ in single C2C12 cell treated with LPA

Marius *et al.* [36] have reported that Ca^{2+} release from the nucleoplasmic reticulum (NR) plays an important role in the Ca^{2+} mobilization in C2C12 cells. To investigate whether LPA has an effect on Ca^{2+} concentration in nucleus, cells were loaded with fluo-3/AM and visualized by confocal microscopy. The fluo-3/AM dye was seen to be highly localized in the nucleus under resting conditions. This could be a reflection of either more $[Ca^{2+}]_i$ in the nucleus than in the cytosol or compartmentalization of the dye to the nucleus. It is pointed out that a similar observation has been reported in cultured cortical astrocytes [37]. Exogenous LPA caused a significant increase in the Ca^{2+} concentration in both the nucleus and cytoplasm (Fig. 5). The peak response was observed at 30 sec after addition of LPA.

Table 2 Effect of different agents on Ca^{2+} release due to LPA treatment of C2C12 skeletal muscle cells

Group	Basal $[Ca^{2+}]_i$ (nM)	LPA-induced increase in $[Ca^{2+}]_i$ above the basal (nM)
DMSO (10 µl)	93 ± 10	69 ± 11
TG (5 µM)	195 ± 20*	63 ± 10
Caffeine (10 mM)	146 ± 12*	54 ± 10
XEC (10 µM)	99 ± 8	66 ± 11
U73122 (1 µM)	101 ± 5	75 ± 11
KCl (30 mM)	123 ± 2*	36 ± 8*

Values are means ± S.E.M. of six different measurements. C2C12 cells were pre-treated with the different agents for 10 min prior to addition of LPA (10 mM). The $[Ca^{2+}]_i$ was measured as described in the Materials and methods. *P<0.05 versus vehicle control (DMSO) group. TG, thapsigargin; XEC, xestospongin.

Effect of LPA on DNA synthesis in C2C12 cells

Intracellular Ca^{2+} signalling plays an important role in gene expression and DNA replication [36]. Since LPA has been observed to increase Ca^{2+} in both the nucleus and cytoplasm, it is possible that LPA might also induce DNA synthesis in C2C12 cells. Accordingly, we determined the effect of LPA on DNA synthesis in C2C12 cells. It can be seen in Figure. 6 that LPA induced an increase in DNA synthesis as evidenced by an increase in $[^3H]$-thymidine incorporation in C2C12 cells, which was prevented by the PI3-K inhibitor, wortmannin (100 nM). Wortmannin alone had no effect on $[^3H]$-thymidine incorporation in C2C12 cells.

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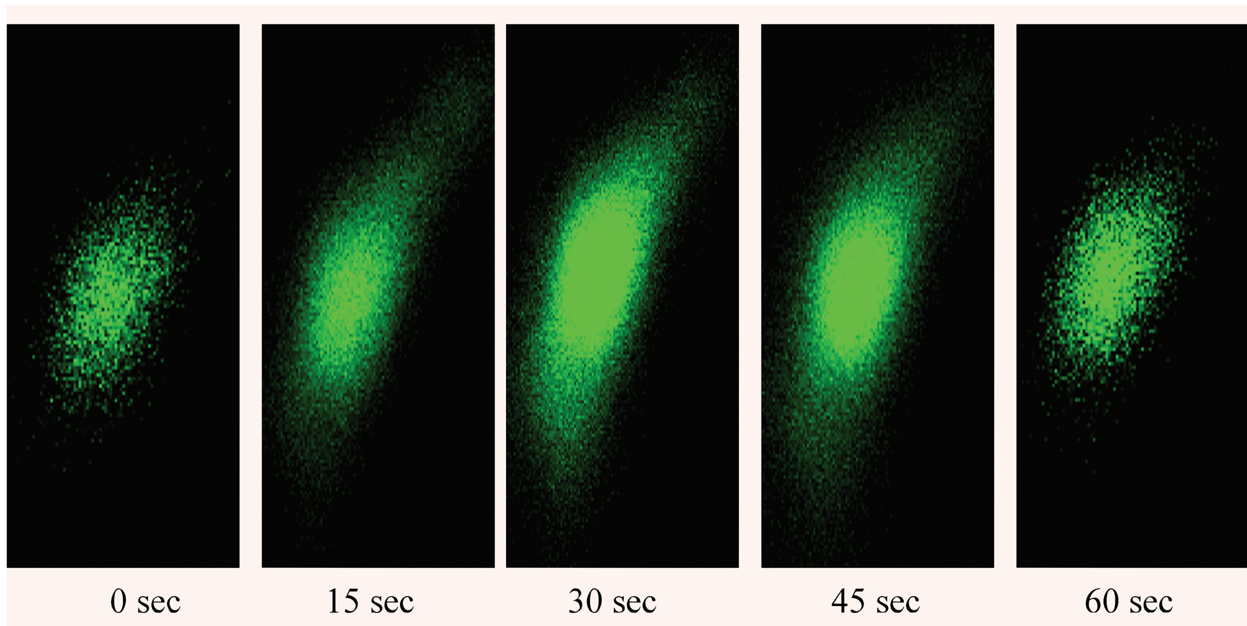

Fig. 5 Lysophosphatidic acid-induced changes in nuclear and cytosolic calcium in single C2C12 cells. The C2C12 cells were loaded with 10 μM fluo-3/AM for 30 min. Cells were washed three times with HEPES buffer and scanned in time series with a Nikon TE2000-U confocal microscope. The $[Ca^{2+}]_i$ is reflected by the intensity of the green fluorescence of fluo-3.

Discussion

Several lines of evidence indicate that LPA is a lipid mediator, abundantly present in the blood, with a diverse range of biological actions including the regulation of $[Ca^{2+}]_i$ and proliferation in different cell types [2, 3, 17–20, 27, 38–41]. In fact, Ca^{2+} is considered to play a critical role in the activation of signal transduction mechanisms involved in cell proliferation [27]. However, there is no information in the literature regarding the effects of LPA on $[Ca^{2+}]_i$ in skeletal muscle. We are the first to report that LPA induces an increase in $[Ca^{2+}]_i$ in a concentration and time dependent manner in C2C12 cells. The LPA-induced increase in $[Ca^{2+}]_i$ was significantly inhibited by three $LPA_{1/3}$ receptor antagonists, as well as with PTX, genistein and tyrphostin A9, suggesting that the LPA-induced increase in $[Ca^{2+}]_i$ is mediated by $LPA_{1/3}$ receptors that may be coupled to G_i protein and tyrosine kinase (Fig. 7). Similar mechanisms of LPA-induced increase in $[Ca^{2+}]_i$ have also been reported in VSMCs and T cells [32, 33]. Exogenous LPA has also been reported to activate PI3-K in cardiomyocytes [21]. PI3-K has been demonstrated to

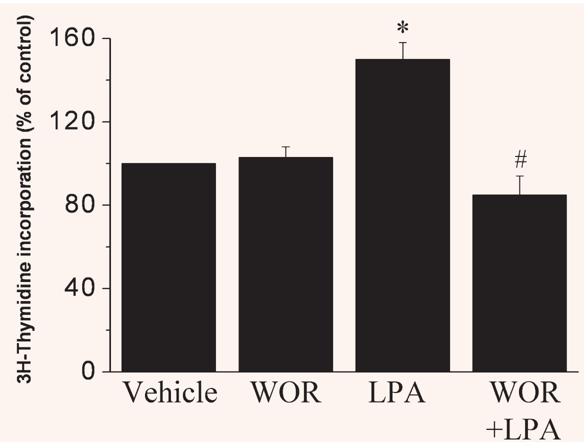

Fig. 6 Effect of lysophosphatidic acid on C2C12 cell proliferation in the absence and presence of phosphatidylinositol 3-kinase inhibition with wortmannin. The C2C12 cell proliferation was determined by the incorporation of $[^3H]$-thymidine into C2C12 cells. 100 nM wortmannin (WOR) was added to the culture medium 10 min prior to the addition of LPA (10 μM). After a 4 hrs incubation with LPA, 1 μCi $[^3H]$-thymidine was added. The reaction was terminated after 22 hrs. Values are means ± S.E.M. of six experiments. **P*<0.05 *versus* vehicle control value; #*P*<0.05 *versus* LPA value in the absence of wortmannin.

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regulate Ca^{2+} signalling in T cells [17]. In the present study, pre-treatment of C2C12 cells with PI 3K inhibitors wortaminnin and LY 294002, significantly attenuated the Ca^{2+} response to LPA in C2C12 cells, indicating that PI3-K may be involved in Ca^{2+} mobilization in skeletal muscle. In view of the reported activation of PI3-K through Gi-protein in leukotriene β-induced degranulation in rat basophilic leukaemic cells [42], it is possible that the $LPA_{1/3}$ receptor may transduce its signal to PI3-K *via* G_i-protein in skeletal muscle. However, an interaction between tyrosine kinase and PI3-K cannot be excluded and therefore warrants further investigation (Fig. 7). The partial inhibition observed with $LPA_{1/3}$ receptor antagonists is suggestive of an involvement of other LPA receptors in the increase in $[Ca^{2+}]_i$ in response to LPA. Our preliminary data have revealed that higher concentrations of DGPP (50 and 100 μM) did not abolish the LPA-induced increase in $[Ca^{2+}]_i$, indicating that indeed other LPA receptor subtypes could be involved in the LPA response.

We have previously reported that EGTA significantly reduces the LPA-induced increase in $[Ca^{2+}]_i$ in VSMCs and have suggested that the LPA-induced increase in $[Ca^{2+}]_i$ may result from Ca^{2+} influx from extracellular space [26]. Furthermore, we have shown that the L-type Ca^{2+} channel blockers, verapamil and diltiazem, did not produce any significant change in the basal and LPA-mediated increase in $[Ca^{2+}]_i$ in VSMCs [26, 27]. Likewise, these blockers also had no significant effect on basal and LPA-induced increase in $[Ca^{2+}]_i$ in C2C12 cells. In addition, EGTA (1 mM) reduced both basal as well as peak $[Ca^{2+}]_i$ response evoked by LPA $[Ca^{2+}]_i$. Thus, similar to our earlier findings in VSMCs [26, 27], LPA does not appear to induce Ca^{2+} entry from the extracellular source through the L-type Ca^{2+} channels in skeletal muscle cells. LPA induced Ca^{2+} influx has been reported to occur *via* SOCs in VSMCs [26]. In the present investigation, SOCs blockers, Gd^{3+} and La^{3+} [25, 36] did not exert any significant effect on LPA-evoked increase in $[Ca^{2+}]_i$, indicating that Ca^{2+} mobilization by LPA does not involve extracellular Ca^{2+} entry through SOCs. A similar observation has also been reported in human neutrophils [20].

The role of Na^+–Ca^{2+} exchanger on LPA-induced increase in $[Ca^{2+}]_i$ in C2C12 cells was also investigated by pre-treating cells with two Na^+–Ca^{2+} exchanger inhibitors, Ni and KB-R7943 [26]. These two agents inhibited the LPA-initiated response by 40–55%, indicating that LPA may induce Ca^{2+} influx through Na^+–Ca^{2+} exchanger. Similar results have been reported in VSMCs where an attenuation of the LPA-induced increase of $[Ca^{2+}]_i$ was observed in the presence of Na^+–Ca^{2+} exchanger inhibitors [26] suggesting that Ca^{2+} entry through Na^+–Ca^{2+} exchanger may be a common mechanism contributing to the LPA-induced increase $[Ca^{2+}]_i$ in both VSMCs and C2C12 skeletal muscle cells. Tokumura *et al.* [43] have reported that the LPA-induced activation of Na^+–Ca^{2+} exchanger is attenuated by tyrosine kinase inhibition in cultured bovine adrenal

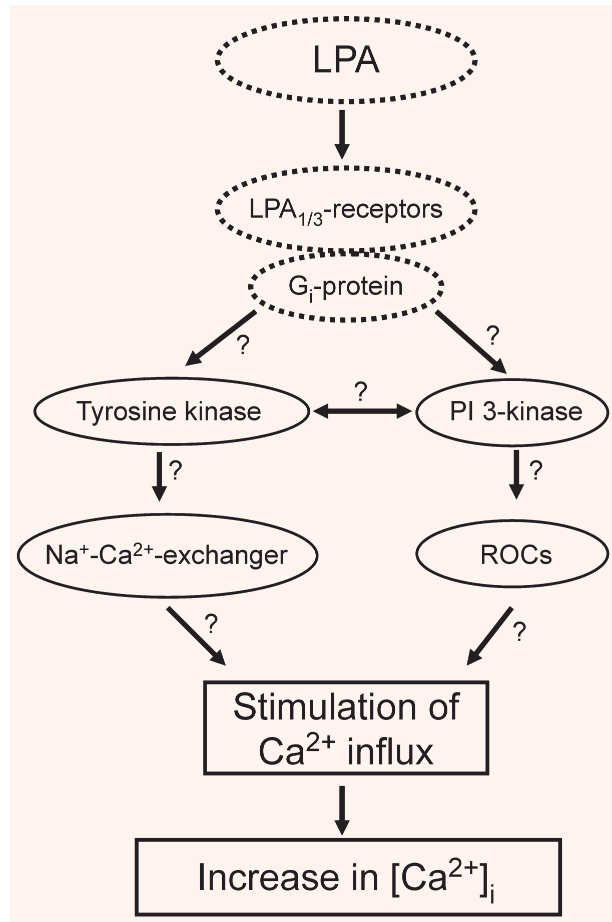

Fig. 7 Schematic representation of the hypothetical mechanisms involved in lysophosphatidic acid-induced increase in intracellular calcium in C2C12 cells. LPA: lysophosphatidic acid, PI3-K: phosphatidylinositol 3-kinase, ROCs: receptor operated channels, $[Ca^{2+}]_i$: intracellular calcium concentration.

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chromaffin cells. In the present study, the increase of $[Ca^{2+}]_i$ induced by LPA in C2C12 cells was attenuated not only with Na^+–Ca^{2+} exchanger inhibitors, but also with inhibition of tyrosine kinase activity. Thus, it would be interesting to examine if tyrosine kinase is involved in the regulation of Na^+–Ca^{2+} exchanger activity in skeletal muscle cells (Fig. 7).

Although ROCs play an important role in Ca^{2+} influx in T cells [44], their role in C2C12 cells have not been examined. Recently it has been reported that transient receptor potential (TRP) C7 cation channels are inhibited by 2-APB [44, 45]. In the present study pre-treatment of C2C12 cells, with SK&F 96365, a ROC blocker [39] and (TRP) C7 cation channels blocker 2-APB [42] significantly attenuated the LPA-induced increase in $[Ca^{2+}]_i$ by 50–60%. These results indicate that unlike that in VSMCs, where Ca^{2+} mobilization by LPA involves extracellular Ca^{2+} influx through SL SOCs, Ca^{2+} mobilization from extracellular by LPA involves ROCs in skeletal muscles. It has been reported that ROCs are linked to G-proteins [47]. Furthermore, Su *et al.* [48] have shown that activation of PI3-K is involved in Ca^{2+} dependent signalling at the level of ROCs in swine carotid arteries. Since ROCs have been reported to play an important role in Ca^{2+} and that LPA activates PI3-K [12, 21], which is known to regulate Ca^{2+} signalling in T cells [17], it is apparent that the LPA-induced Ca^{2+} influx in skeletal muscle cells may involve ROCs through G-protein linked PI3-K ancillary to Na^+-Ca^{2+} exchanger (Fig. 7).

Various studies have demonstrated that there are several Ca^{2+} stores in the SR, and that different stimuli may cause Ca^{2+} release from IP3-, caffeine- or KCl-sensitive SR stores [49–51]. Subsequent experiments were designed to delineate the source of intracellular Ca^{2+} release contributing to the LPA-induced increase in $[Ca^{2+}]_i$ in C2C12 cells. While the LPA-induced increase in $[Ca^{2+}]_i$ in VSMCs has been shown to be mediated by PLC and IP_3 [26], a similar role for PLC and IP_3 in the LPA-evoked $[Ca^{2+}]_i$ response in C2C12 cells was not evident In this study, no significant inhibition on the LPA evoked increase in $[Ca^{2+}]_i$ was found when C2C12 cells were pre-incubated with U73122, a PLC inhibitor and XEC, a membrane permeable IP_3 receptor blocker and thapsigargin a depleter of IP3 sensitive SR Ca^{2+} store [26, 52]. These results suggest that an IP_3-dependent intracellular Ca^{2+}-store may not be involved in the increase in $[Ca^{2+}]_i$ due to LPA in C2C12 cells. While the LPA-induced increase in $[Ca^{2+}]_i$ in human neuroblastoma cells has also been reported to occur independently of IP3 or ryanodine-receptors [53], the mechanism of LPA-induced increase in $[Ca^{2+}]_i$ in VSMCs involves the IP3-sensitive intracellular Ca^{2+} pool and activation of PLC [26]. The vasopressin-induced Ca^{2+} mobilization in neonatal rat cardiomyocytes has been reported to occur in a PLC-dependent manner and independent of the ryanodine- or caffeine-sensitive intracellular Ca^{2+}-stores, suggesting that there are two SR Ca^{2+}-pools, one a ryanodine-sensitive and the other an IP3- Ca^{2+} sensitive pool [49]. In the present study, caffeine alone was found to cause a significant increase in $[Ca^{2+}]_i$, but it did not produce any effect on the LPA-induced increase in $[Ca^{2+}]_i$ in C2C12 cells. These data indicate that LPA may not induce Ca^{2+} release from caffeine sensitive SR store.

Lorenzon *et al.* [54] have recently demonstrated that depolarization of C2C12 cells by KCl evokes Ca^{2+} release from SR through non-caffeine-sensitive receptors, indicating the presence of voltage- and ligand-gated ryanodine receptors on SR in differentiated C2C12 cells. Some reports have also indicated that ryanodine receptor response to caffeine, voltage or ryanodine is functionally different [55, 56]. The effect of membrane depolarization by KCl in the LPA-induced response in C2C12 cells was also examined. Pre-treatment of C2C12 cells with 30 mM KCl alone induced a small, but significant increase in the basal $[Ca^{2+}]_i$; however, the LPA-induced increase of $[Ca^{2+}]_i$ was inhibited by about 50%. These data suggest that LPA may induce Ca^{2+} release through voltage-gated ryanodine receptors from SR or NR. On the other hand, membrane depolarization by KCl is known to deplete SR Ca^{2+} stores and suppress Ca^{2+} entry [57] resulting in morphological and functional changes in the mitochondrial permeability transition pore [58], it is also possible that membrane depolarization by KCl may change the structural configuration of ROCs or Na^+–Ca^{2+} exchanger and inactivate ROCs and Na^+–Ca^{2+} exchanger. Although we have earlier reported a positive inotropic effect of LPA in rat heart *in vivo* [59], such a response in skeletal muscle is not known; however, in view of the critical importance of $[Ca^{2+}]_i$ in skeletal muscle contractile activity [22–25], it is likely that LPA would regulate a contractile response in skeletal muscle. Nevertheless, the functional

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relevance of the LPA effects in C2C12 cells was addressed by assessment of DNA synthesis in response to LPA. We observed an increase in DNA synthesis as evidenced by the increase in [^{3}H] thymidine incorporation due to LPA. Since wortmannin prevented the LPA-induced DNA synthesis in C2C12 cells, it can be suggested that PI3-K has an important role to play in LPA signal transduction mechanisms and skeletal muscle cell proliferation. In fact, the increase in nuclear Ca^{2+} in the C2C12 cells in response to LPA, observed in the present study, may serve as a signal for DNA synthesis [36] and thus may be related to cell proliferation. This aspect warrants further investigation.

Conclusion

Our findings suggest that LPA causes a time and concentration dependent increase in $[Ca^{2+}]_i$ which is dependent on extracellular Ca^{2+} concentration involving Ca^{2+} influx through Na^+–Ca^+ exchanger and ROCs in skeletal muscle cells but not SOCs as observed by Gutierrez-Martin *et al.* [60]. This action seems to be mediated through G_i-protein linked $LPA_{1/3}$ receptors, which in turn activates tyrosine kinase and PI3-K (Fig. 7). However, further studies are required to confirm the link between the tyrosine kinase and PI3-K with Na^+–Ca^+ exchanger and ROCs. The present study also demonstrated that the mechanisms of LPA-induced increase in $[Ca^{2+}]_i$ may depend upon cell type. The limitation of the present study includes the fact that the observations from cultured C2C12 cells may not represent the physiological response to LPA in skeletal muscle cells under *in vivo* conditions. Although the physiological role of the LPA-induced increase in $[Ca^{2+}]_i$ in C2C12 cells is remains to be completely understood, the results of the present study provide novel information on the mechanisms of LPA-induced increase in the cytosol and nuclear $[Ca^{2+}]$ as well as DNA synthesis in skeletal muscle cells. The increase in $[Ca^{2+}]_i$ due to LPA may serve as the trigger for the activation of signal transduction processes that ultimately result in cell proliferation. However, a direct relation between the increases in $[Ca^{2+}]_i$ and cell proliferation remains to be determined. Furthermore, the link between the changes in the nucleus *versus* cytosolic $[Ca^{2+}]_i$ also remains to be established. Nevertheless, our data suggest that LPA could play an important role in the regulation of skeletal muscle $[Ca^{2+}]_i$ and cell proliferation, which may lead to a novel treatment for skeletal muscle dystrophy.

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