Vol. 208, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 8, 1995 Pages 19-25

SENSITIZING EFFECT OF LYSOPHOSPHATIDIC ACID ON MECHANORECEPTOR-LINKED RESPONSE IN CYTOSOLIC FREE Ca^{2+} CONCENTRATION IN CULTURED SMOOTH MUSCLE CELLS

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Received January 23, 1995

Summary: We found that lysophosphatidic acid (LPA) sensitizes response in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) to mechanical stimulation in cultured longitudinal muscle cells from guinea pig ileum. $[Ca^{2+}]_i$ was transiently increased by spritzing of bath solution onto cells as mechanical stimulation in the presence of LPA , but not in absence of LPA. The effect was reversible and concentration-dependent (1-30 nM). Ga^{3+} but not nicardipine inhibited the $[Ca^{2+}]_i$ transient in the presense of LPA. Phosphatidic acid also induced the sensitization, but the effective concentration was more than 10 times higher than in LPA. Histamine and carbachol did not have any sensitizing effect to mechanical stimulation. These results show that LPA sensitizes mechanoreceptor-linked response, suggesting that LPA may play an important role in mechanotransduction mechanisms as an endogenous regulatory factor.

Lysophosphatidic acid (LPA), simplest naturally occurring phospholipids, are produced rapidly in significant quantities by cell activation (1-3), suggesting a possible role of LPA as second messengers. Additionally, application of LPA to cell induces various biological effects in several cell types (4, 5). It was also shown that LPA is newly formed and released from thrombin-activated platelets into the extracellular environment (6). Recently, a putative receptor of LPA was identified by binding assay (7). Therefore, LPA may act as not only second messengers but autocrine or paracrine. Thus, LPA may be very important for regulation of various cellular response, but LPA is not still established as signaling molecule. In this view point, when we were examining effect of LPA on cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) which is key signal of various cellular responses to clarify molecular mechanisms of LPA-induced cellular effects, we found that addition of LPA to cultured smooth muscle cells sensitizes response in $[Ca^{2+}]_i$ to mechanical stimulation.

Most cells can expose to several types of mechanical stress from extracellular environment and adjacent cells in multicellular animals. It is considered that mechanoreceptor can exist not only in sensory organs but in various tissues such as heart, intestine, urinary bladder, lung and blood vessel, and always monitor their contraction, relaxation and extension. Subsequently, cells exposed to mechanical stress can cause various cellular responses through mechanotransduction mechanisms (8-10). Therefore, mechanoreceptor may play an important

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0006-291X/95 $5.00

role in maintenance of cellular homeostasis and cell-to-cell interaction, but the molecular mechanisms remain unclear.

In the present study, to clarify whether LPA acts as an endogenous regulatory factor of mechanotransduction mechanisms, properties of the sensitization induced by LPA were investigated.

MATERIALS AND METHODS

Materials: Fluo-3 acetoxymethyl ester (fluo-3/AM) and 1,2-Bis(2-amino-5-fluorophenoxy) ethan-N,N,N',N'-tetraacetic acid (BAPTA) were obtained from Dojin Laboratories. Lysophosphatidic acid (LPA: from egg yolk lecithin), phosphatidic acid (PA: from egg yolk lecithin) and lysophosphatidylcholine (LPC: oleoyl) were purchased from Avanti Polar Lipids, Inc. Nicardipine was obtained from Sigma Chemical Co. All other chemicals were commercial products of the highest available grade of purity.

Cell culture: The cultured longitudinal muscle cells were obtained from guinea pig ileum as described previously (11). The longitudinal muscle was carefully stripped off and placed in Tyrode-Hepes solution containing (mM) NaCl, 137; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 1.0; glucose, 5.6; N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), 8.4; pH 7.4. The longitudinal muscle segments were cut into small pieces. Small explants of longitudinal muscle were cultured in minimum essential medium (MEM, Gibco) containing 20% fetal calf serum on 25-mm-diameter glass coverslips inside 35-mm Petri dishes and maintained at 37°C under humidified conditions of 95% air - 5% CO_2. After 24 hr, the culture medium was replaced with MEM containing 10% fetal calf serum, and the medium was changed every 3 days until the cells were grown to complete confluency. The confluent cells were subcultured. The confluent cells of primary and secondary culture used for the experiments were confirmed that they originated from smooth muscle cells by immunofluorescence technique using monoclonal antibody to smooth muscle cells (Boehringer Mannheim GmbH).

Measurement of $[Ca^{2+}]_i$: To determine the $[Ca^{2+}]_i$, the cultured cells on a coverslip were incubated with 5 μM fluo-3/AM in culture medium at 37°C under 5% CO_2 in air for 1 hour. After loading, the coverslips were rinsed several times with Tyrode-Hepes solution. After washing, the coverslip was mounted in an experimental chamber. Fluorescence images of fluo-3 were collected under the various conditions using a Biorad MRC-500 confocal laser scanning attachment mounted on a Nikon Diaphot inverted microscopy. Excitation wavelength of 488 nm was provided by an Argon laser and was attenuated with 1% neutral density filter to minimize photobleaching and photodamage. Green fluorescence of fluo-3 was collected using a 510-nm long pass dichroic reflector and a 515-nm long pass emission filter. The temperature was kept at 32°C, since intracellular fluorescence of fluo-3 decreased rapidly at 37°C, probably by dye leakage. The mean intensity of fluo-3 fluorescence for each individual cell was obtained by averaging the intensity of each pixel within a rectangular zone centered over the cell.

Application of mechanical stimulation: Bath solution was perpendicularly spritzed onto fluo-3-roaded cells from pipette at an appropriate constant flow rate (1 - 6.7 ml/min) for 3 seconds. Tip of the pipette was settled at 2 mm right over the interest cells and the inside diameter of the tip was 1 mm. This mechanical stimulation did not affect acquisition of the images of fluo-3 fluorescence.

RESULTS

Fig. 1 shows time course of changes in $[Ca^{2+}]_i$ to mechanical stimulation by spritzing bath solution at 1.8 ml/min of flow rate onto the cultured longitudinal muscle cells in the absence and the presence of 30 nM LPA from egg yolk lecithin. In the resting state, the mechanical stimulation did not cause significant change in $[Ca^{2+}]_i$ as shown in Fig. 1A, although spontaneous oscillation of $[Ca^{2+}]_i$ was observed in about 50% of the cells as

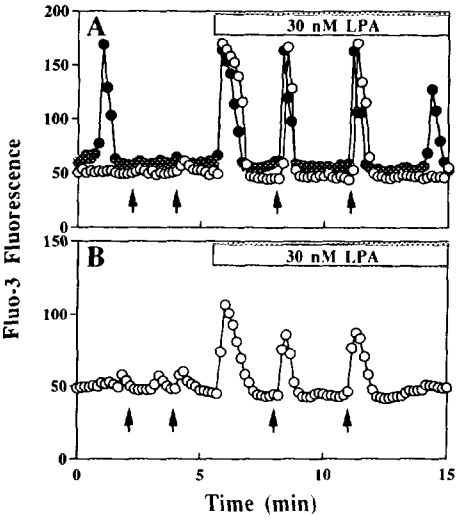

Fig. 1. Effect of LPA on changes in $[Ca^{2+}]_i$ to mechanical stimulation in cultured smooth muscle cells. 30 nM LPA was added to the cells as shown at the top of the panel. Arrows show mechanical stimulation by spritzing bath solution onto the cells at 1.8 ml/min of flow rate as described in MATERIALS AND METHODS. A: Closed and open circles represent two different cells in the same microscopic field. B: Averaged data from 30 cells in the same microscopic field.

described previously (11). Addition of 30 nM LPA induced a transient increase in $[Ca^{2+}]_i$. After the $[Ca^{2+}]_i$ returned to baseline level, the same mechanical stimulation with that in the resting state caused a transient increase in $[Ca^{2+}]_i$. Namely, the $[Ca^{2+}]_i$ began to increase after a few second of lag time from the mechanical stimulation, reached to peak within 20 second after the stimulation, and suddenly fell to the baseline level within 1 min after the stimulation. Subsequent mechanical stimulation caused the same increase in $[Ca^{2+}]_i$ as in the first stimulation. Washout of LPA abolished the effect. This sensitizing effect of LPA occurred in more than 70% of mechanically stimulated cells. Averaged data from 30 cells shown in Fig. 1B indicates that the $[Ca^{2+}]_i$ transients occurred almost simultaneously in the most cells and that the transients induced by the second mechanical stimulation were same as those in the first stimulation.

We then examined dose-response relationship of the $[Ca^{2+}]_i$ transients to mechanical stimulation by changing flow rate of spritzing onto the cells as shown in Fig. 2. In the absence of LPA, the stimulation did not cause significant response at less than 3.4 ml/min of flow rate, and when we defined responded cell to the mechanical stimulation as cell that increases fluorescence of fluo-3 to more than 30% of baseline level within 30 second after the mechanical stimulation, percentage of responded cells was less than 30% even at 6.7 ml/min. In the presence of 30 nM LPA, 60 % of the cells responded to the stimulation at 1.4 ml/min. Namely, LPA decreased the degree of the mechanical stimulation to induce transient increase in $[Ca^{2+}]_i$, and also increased percentage of responded cells to the mechanical stimulation, indicating that LPA sensitizes response in $[Ca^{2+}]_i$ to mechanical stimulation in the cultured smooth muscle

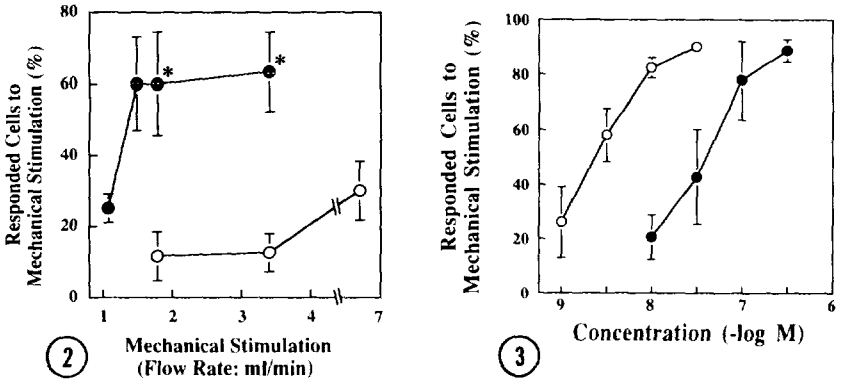

Fig. 2. Dose-response relationship between percentage of responded cells to mechanical stimulation and degree of mechanical stimulation in the absence and the presence of LPA. Cells were mechanically stimulated by spritzing of bath solution at each flow rate before (open circle) and after adding 30 nM LPA (closed circle). We defined responded cell to the mechanical stimulation as cell that increases fluorescence of fluo-3 to more than 30% of baseline level within 30 seconds after the mechanical stimulation. Percentages of responded cells to each mechanical stimulation were plotted as the mean ± S.E. for 3 experiments. Significantly different from the values before adding LPA, *p<0.05.

Fig. 3. Dose-response relationship between percentage of responded cells to mechanical stimulation and concentrations of LPA and PA. Percentages of responded cells to mechanical stimulation at 1.8 ml/min of flow rate in the presence of each concentration of LPA (open circle) and PA (closed circle) were plotted as the mean ± S.E. for 3 experiments.

cells. We confirmed that synthesized LPA (oleoyl) has the same effect as LPA from egg yolk lecithin (data not shown). The effect of LPA was concentration-dependent manner and the effective concentration (EC_{50}) where 50% of the cells responds to mechanical stimulation was about 2.5 nM as shown in Fig. 3. Also we examined effect of PA, one of Ca^{2+}-mobilizing phospholipids (12-14), on the response in $[Ca^{2+}]_i$ to mechanical stimulation in the same condition. PA caused the same effect as LPA in concentration-dependent manner, but the EC_{50} was about 40 nM in the cultured longitudinal muscle cells as shown in Fig. 3.

To elucidate mechanisms for increase in $[Ca^{2+}]_i$ to mechanical stimulation in the presence of LPA, we examined effects of removing extracellular Ca^{2+} and two types of channel blockers on the $[Ca^{2+}]_i$ transient. As shown in Fig. 4, the $[Ca^{2+}]_i$ transient was not caused by mechanical stimulation after adding 2.1 mM BAPTA to the Tyrode-Hepes solution containing 30 nM LPA to reduce concentration of extracellular Ca^{2+} to about 2.6 μM. In addition, 10 μM Gd^{3+}, a blocker for cation-selective mechanosensitive channels (15), inhibited the $[Ca^{2+}]_i$ transient, although 10 μM nicardipine, a blocker for voltage-dependent (L-type) Ca^{2+} Channels, did not have significant effect on the $[Ca^{2+}]_i$ transient.

To clarify whether the sensitizing effect is specific to LPA or not, we then examined effects of biologically active lysophospholipid, LPC (16, 17), and Ca^{2+}-mobilizing agonists, histamine (His) and carbachol (CCh) on Ca^{2+} response to the mechanical stimulation. As shown in Fig. 5, LPC caused the same sensitizing effect as LPA, but the EC_{50} is about 3 μM which is about 1000 times higher than that in LPA. On the other hand, 1 μM His and 10 μM CCh did not have significant effect.

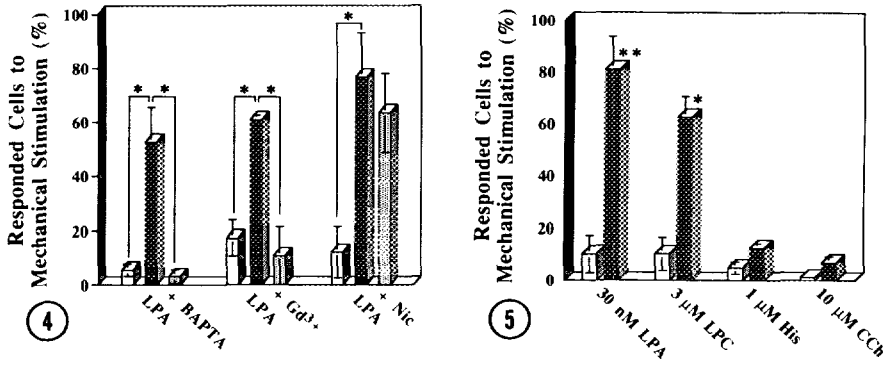

Fig. 4. Effects of removing extracellular Ca^{2+} and adding Gd^{3+} and nicardipine on $[Ca^{2+}]_i$ response to mechanical stimulation in the presence of LPA. Percentages of responded cells to mechanical stimulation at 1.8 ml/min of flow rate before (open column) and after adding 30 nM LPA (closed column), and after adding 2.1 mM BAPTA, 10 μM Gd^{3+}, and 10 μM nicardipine (+ Nic), in the presence of 30 nM LPA were represented as the mean ± S.E. for 3 experiments, respectively. Significantly differences, *p<0.05.

Fig. 5. Effects of LPA, LPC, His and CCh on $[Ca^{2+}]_i$ response to mechanical stimulation. Percentages of responded cells to mechanical stimulation at 1.8 ml/min of flow rate before (open column) and after each treatment (closed column) were represented as the mean ± S.E. for 3 experiments. Significantly different from the values before each treatment, *p<0.05, **p<0.01.

DISCUSSION

It has been known that mechanical stress caused a transient increase in $[Ca^{2+}]_i$ in several cell types (18-20), and it is considered that mechanosensitive ion channel is involved in the response. However, the molecular mechanisms remain unclear and also any endogenous regulatory factor of the mechanisms has not been known.

In the present study, we found that LPA sensitizes response in $[Ca^{2+}]_i$ to mechanical stimulation in concentration-dependent manner in cultured smooth muscle cells. Mechanical stimulation-induced $[Ca^{2+}]_i$ transient in the presence of LPA was inhibited by either removing extracellular Ca^{2+} or adding 10 μM Gd^{3+}, a blocker for cation-selective mechanosensitive channels (15), but not by adding 10 μM nicardipine, a blocker for voltage-dependent (L-type) Ca^{2+} channels (Fig. 3). These properties of the $[Ca^{2+}]_i$ transient closely resembled those in stretch-induced $[Ca^{2+}]_i$ increase, which may arise from Ca^{2+} entry through stretch-activated channels, in endothelial cells (19). Therefore, our results suggest that LPA sensitizes mechanical stimulation-induced $[Ca^{2+}]_i$ response related to Ca^{2+} entry through Gd^{3+}-sensitive channels, probably stretch-activated channels. The possibility that Ca^{2+} release from intracellular store sites relates to the $[Ca^{2+}]_i$ transient is now under investigated.

On the other hand, it was considered that there is possibility that the sensitizing effect of LPA may result from it's nonspecific action to $[Ca^{2+}]_i$ as Ca^{2+}-mobilizing substances. To test this possibility, we examined the effects of His and CCh, Ca^{2+}-mobilizing agonists, on response in $[Ca^{2+}]_i$ to mechanical stimulation. From the results that His and CCh did not have

the sensitizing effect, it was confirmed that the sensitizing effect of LPA is not due to it's nonspecific action as Ca^{2+}-mobilizing substances. On the other had, also LPC caused the sensitizing action at the high concentration (3 μM). This effect may be due to its detergent action which alters fluidity of cell membrane and subsequently influences function of a number of membrane receptors and channels (21). However, it is unlikely that the effect of LPA is the same detergent action as LPC, from the result that the EC_{50} for LPC was about 1000 times higher than that for LPA. We considered that very low value of EC_{50} (2.5 nM) for LPA in the sensitizing effect supports that it is a specific action of LPA. In fact, the EC_{50} for LPA corresponded with affinities of a specific binding sites for LPA in membranes prepared from rat brain and Swiss 3T3 fibroblasts (7), suggesting a possibility that the sensitizing effect is caused via binding of LPA to it's receptor. The weak sensitizing effect of PA may be caused via LPA receptor, because also PA can bind LPA receptor (7). The sensitizing effect of LPA may relate to the demonstration that nanomolar concentrations of LPA induce the rapid formation of actin stress fibres and assembly of focal adhesions in serum-starved Swiss 3T3 cells (22), because it is considered that mechanosensitive channel must be connected to the cytoskeleton (23). From above discussion, it is concluded that LPA has sensitizing effect on response in $[Ca^{2+}]_i$ to mechanical stimulation as it's specific action, although it is unclear whether the effect results from binding of LPA to it's receptor. This is the first finding showing a possibility that mechanoreceptor-linked Ca^{2+} response can be regulated by endogenous substance. Although further studies are required to elucidate action site and manner of LPA in the sensitizing effect, also the elucidation may help clarification of mechanotransduction mechanisms.

What is the physiological role in the sensitization of mechanoreceptor-linked Ca^{2+} response by LPA in longitudinal muscle cells? Intestinal smooth muscle has spontaneous rhythms of electrical and mechanical activity (24), but the mechanisms remain unclear. There is a possibility that LPA modifies sensitivity of monitoring their contraction and relaxation to regulate the intestinal mechanical function. Furthermore, if the sensitization by LPA occurs in cell types other than intestinal longitudinal muscle cells, for example endothelial cells and various epithelial cells, LPA may play an important role in monitoring mechanical stress from extracellular environment and adjacent cells. This possibility is now under investigated.

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