

Activation of Lysophosphatidic Acid Receptor Is Coupled to Enhancement of Ca^{2+} -Activated Potassium Channel Currents

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The calcium-activated K^+ (BK_{Ca}) channel is one of the potassium-selective ion channels that are present in the nervous and vascular systems. Ca^{2+} is the main regulator of BK_{Ca} channel activation. The BK_{Ca} channel contains two high affinity Ca^{2+} binding sites, namely, regulators of K^+ conductance, RCK1 and the Ca^{2+} bowl. Lysophosphatidic acid (LPA, 1-radyl-2-hydroxy-*sn*-glycero-3-phosphate) is one of the neurolipids. LPA affects diverse cellular functions on many cell types through G protein-coupled LPA receptor subtypes. The activation of LPA receptors induces transient elevation of intracellular Ca^{2+} levels through diverse G proteins such as $\text{G}\alpha_{\text{q/11}}$, $\text{G}\alpha_{\text{i}}$, $\text{G}\alpha_{12/13}$, and $\text{G}\alpha_{\text{s}}$ and the related signal transduction pathway. In the present study, we examined LPA effects on BK_{Ca} channel activity expressed in *Xenopus* oocytes, which are known to endogenously express the LPA receptor. Treatment with LPA induced a large outward current in a reversible and concentration-dependent manner. However, repeated treatment with LPA induced a rapid desensitization, and the LPA receptor antagonist Kil6425 blocked LPA action. LPA-mediated BK_{Ca} channel activation was also attenuated by the PLC inhibitor U-73122, IP_3 inhibitor 2-APB, Ca^{2+} chelator BAPTA, or PKC inhibitor calphostin. In addition, mutations in RCK1 and RCK2 also attenuated LPA-mediated BK_{Ca} channel activation. The present study indicates that LPA-mediated activation of the BK_{Ca} channel is achieved through the PLC, IP_3 , Ca^{2+} , and PKC pathway and that LPA-mediated activation of the BK_{Ca} channel could be one of the biological effects of LPA in the nervous and vascular systems.

Key Words: BK_{Ca} channels, Ca^{2+} signal pathways, LPA, LPA receptor, *Xenopus* oocytes

INTRODUCTION

Calcium-activated K^+ (BK_{Ca}) channels are high-conductance, voltage-dependent, and potassium-selective ion channels. BK_{Ca} channels are activated by depolarization and elevation of intracellular Ca^{2+} levels and causes a closure of voltage-dependent Ca^{2+} channels and reduces Ca^{2+} influx into the cell. Thus, BK_{Ca} channels are dually regulated through membrane voltage and intracellular Ca^{2+} . BK_{Ca} channels comprise two subunits, α and β [1-3]. Ca^{2+} -dependent kinases also can phosphorylate the α subunit and regulate the channel activity [4]. BK_{Ca} channels participate in diverse physiological processes such as neuronal


excitability, smooth muscle contractility, hair cell tuning, and have a protective mechanism against ischemic cell death of neurons [5]. In addition, there are two regulators of K^+ conductance, the RCK1 and RCK2 domains in the cytosolic domain of the BK_{Ca} channel [6]. These contain two high affinity Ca^{2+} binding sites. One is in the RCK1 domain at position Asp362/367 and the other is in the Ca^{2+} bowl that contains a series of Asps located in the RCK2 domain [7,8] (Fig. 1A).

Lysophosphatidic acid (LPA, 1-radyl-2-hydroxy-*sn*-glycero-3-phosphate) is the prototypical member of the family of lipid mediators [9] (Fig. 1B). The endothelial gene (EDG) family encodes for the GPCR-specific LPA and S1P [9]. LPA activates diverse cellular actions through interactions with the G protein-coupled LPA receptors, which are responsible for the transient elevation of intracellular Ca^{2+} levels [9-11]. In addition, it was demonstrated that neurite retraction and cell rounding in cell lines of neural origin are caused by LPA [12]. Applying LPA extracellularly induces cell polarization, directional motility, and metabolic burst in human neutrophils [13]. However, relatively little is known about how LPA regulates ion channel activity, especially in Ca^{2+} -dependent ion channels.

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ABBREVIATIONS: LPA, lysophosphatidic acid; GPCR, G protein-coupled receptor; BK_{Ca} , calcium-activated K^+ channel; RCK, regulating the conductance of K^+ .

In the present study, we examined the effects of LPA on BK_{Ca} (rat brain *rSlo*) channel activities expressed in *Xenopus laevis* oocytes by using the two-electrode voltage clamp technique. We found that LPA activates the BK_{Ca} channel in a concentration- and voltage-dependent manner. Moreover, when we treated the cells with LPA repeatedly, we could observe the desensitization of LPA-mediated BK_{Ca} channel activation. By using various inhibitors of phospholipase C, IP₃ receptor, and protein kinase C, we examined the signaling pathway in LPA-mediated BK_{Ca} channel activation. In addition, the effects of site-directed mutagenesis on RCK1 and mutations in the Ca²⁺ bowl in LPA-mediated BK_{Ca} channel activation were examined. Finally, we have discussed the physiological or pharmacological roles of LPA in the nervous and vascular systems.

METHODS

Materials

We used previously reported cDNA for the rat brain BK_{Ca} channel (Gene bank ID: NM_000218) [14]. Lysophosphatidic acid (LPA, 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate, 18 : 1) was purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA). LPA was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C as a stock. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of *Xenopus* oocytes

Xenopus laevis frogs were purchased from *Xenopus* I (Ann Arbor, MI, USA). All procedures for their care and handling were approved by the Konkuk University Institutional Animal Care and Use Committee. To isolate oocytes, frogs were anesthetized with an aerated solution of 3-aminobenzoic acid ethyl ester. Ovary was removed and frogs are sacrificed under anesthetized. Oocytes were separated by treatment with collagenase and agitated for 2 h in a CaCl₂-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), 2.5 mM sodium pyruvate, 100 units penicillin/ml, and 100 μg streptomycin/ml. Stage V~VI oocytes were collected and stored in ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES, pH 7.5) supplemented with 50 μg gentamycin/ml [15]. The oocyte-containing solution was maintained at 18°C with continuous gentle shaking and daily medium changes [16]. All electrophysiological experiments were performed within 3 to 4 days following isolation of the oocytes.

cRNA preparation of BK_{Ca} channels and microinjection

BK_{Ca} channel-encoding cRNAs were prepared using an mMessage mMachine transcription kit (Ambion, Austin, TX, USA) with T7 RNA polymerase. The cRNAs were injected into the animal or vegetal pole of each oocyte one day after isolation. The cRNA was dissolved in RNase-free water at a final concentration of approximately 1 μg/μl, divided into aliquots, and stored at -80°C until further use. BK_{Ca} channel cRNAs (40 nl) were injected into the animal or vegetal pole of each oocyte using a 10 μl VWR microdispenser (VWR Scientific, West Chester, PA, USA). The

injection pipette was made by pulling from glass capillary tubing used for recording electrodes and breaking the tip into a 15~20 μm diameter [16].

Site-directed mutagenesis of the BK_{Ca} α subunit and *in vitro* transcription of BK_{Ca} channel cDNAs

Single amino acid substitutions of the BK_{Ca} channel (Fig. 1B) were made using a QuikChange™ XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), *Pfu* DNA polymerase, and sense and antisense primers encoding the desired mutations. Overlap extension of the target domain by sequential polymerase chain reaction (PCR) was carried out according to the manufacturer's protocol. The final PCR products were transformed into *E. coli* strain DH5α, screened by PCR, and confirmed by sequencing of the target regions. The mutant DNA constructs were linearized at their 3' ends by digestion with *NotI*, and run-off transcripts were prepared using the methylated cap analog, m⁷G(5')-ppp(5')G. The absence of degraded RNA was confirmed by denaturing agarose gel electrophoresis followed by ethidium bromide staining. Similarly, recombinant plasmids containing rat BK_{Ca} channel cDNA inserts were linearized by digestion with the appropriate restriction enzymes, and cRNAs were obtained using the mMessage mMachine *in vitro* transcription kit (Ambion, Austin, TX, USA) with SP6 RNA polymerase or T7 polymerase. The final cRNA products were resuspended at a concentration of 1 μg/μl in RNase-free water and stored at -80°C [16].

Data recording

Data recording for BK_{Ca} channel currents was performed as described by [17]. A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings as pre-

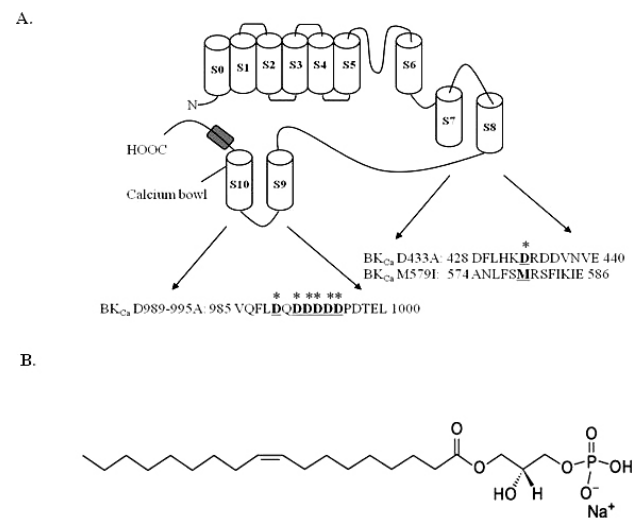


Fig. 1. Schematic transmembrane diagram of the BK_{Ca} channel in the plasma membrane and the LPA chemical structure. (A) There are 7 transmembrane domains along with the RCK1 and RCK2 domains. The voltage sensor and pore of the BK_{Ca} channel resembles the responding regions of other voltage-dependent K⁺ channels (Yuan et al., 2010). Amino acid residues underlined and marked (*) indicate mutation sites in this study. (B) The chemical structure of lysophosphatidic acid (LPA, C₁₈:1).

viously reported. The oocytes were impaled with two microelectrodes filled with 3M KCl (0.2~0.7 MΩ), and electrophysiological experiments were carried out at room temperature using an Oocyte Clamp (OC-725C, Warner Instruments, Hamden, CT, USA). Stimulation and data acquisition were controlled with a pClamp 8 (Axon Instruments, Union City, CA, USA). For most electrophysiological experiments, oocytes were perfused initially with Cl⁻ and Ca²⁺-free solution (in mM: 96 NaOH, 2 KOH, 8 Mg-gluconate, 5 HEPES, 5 EGTA, pH 7.4 with methanesulfonic acid) in the presence of a Cl⁻ channel blocker (500 μM anthracene-9-carboxylic acid) [17] to inhibit endogenous Cl⁻ channels. The oocytes were then clamped at a holding potential of -80 mV, membrane potential was depolarized to +40 mV for 400 ms at 10 s intervals, and currents were recorded as otherwise indicated.

The pH was adjusted to 7.0 with 2-[N-morpholino]ethanesulfonic acid. To compare the channel characteristics accurately, an identical set of intracellular solutions was used throughout the experiments. Commercial software packages, which included Clampex 8.0 or 8.1 (Axon Instruments) and Origin 6.1 (OriginLab Corp., Northampton, MA, USA), were used for the acquisition and analysis of macroscopic recording data.

Data analysis

To obtain the concentration-response curve for the LPA effects on the K⁺ currents from the BK_{Ca} channel, the peak amplitudes at different concentrations of LPA were plotted. The current enhancements or inhibitions evoked by drug treatment were analyzed after subtraction of currents elicited by H₂O injection. Origin software (Origin, Northampton, MA, USA) was used to fit the plot to the Hill equation: $y/y_{\max} = [A]^{nH} / ([A]^{nH} + [EC_{50}]^{nH})$, where y represents the peak current at a given concentration of LPA, y_{\max} is the maximal peak current, EC_{50} is the concentration of LPA producing a half-maximal effect, $[A]$ is the concentration of LPA, and nH is the Hill coefficient. All values are represented as mean±S.E.M. Significant differences between mean control and treatment values were determined using the Student's *t*-test, where $p < 0.05$ was considered statistically significant.

RESULTS

Treatment of LPA in oocytes injected with cRNA encoding the BK_{Ca} α subunit elicited a large outward current with a voltage step from -80 to +40 mV with a 400 ms duration at 10-s intervals (Fig. 2A). We next examined the effects of various concentrations of LPA on the BK_{Ca} channel activity. As shown in Fig. 2, the application of LPA on oocytes induced BK_{Ca} channel activation in a concentration-dependent manner. Charybdotoxin and iberiotoxin, highly specific inhibitor of maxi-K channels [18,19], greatly attenuated the BK_{Ca} channel activations induced by LPA (data not shown), indicating that BK_{Ca} channels are functionally working [20]. Oocytes injected with only H₂O elicited a basal outward current (data not shown). LPA activated the BK_{Ca} channel by an average of 761.42±124.10% at 1 μM. The EC₅₀ was 0.12±0.05 μM (Fig. 2Ab). These results indicate that LPA induces BK_{Ca} channel activation.

The effect of LPA on the current-voltage (I~V) relationship was examined. The current response evoked by each

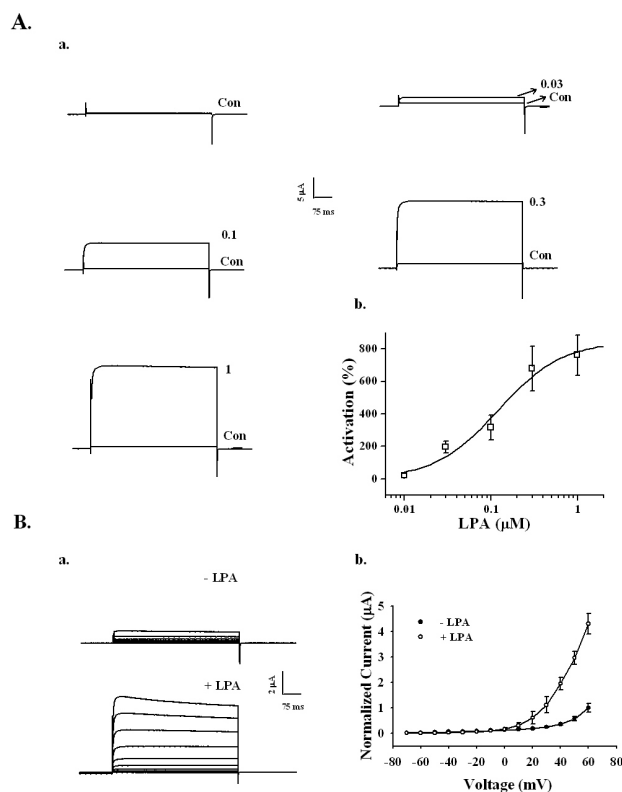


Fig. 2. Concentration- and voltage-dependent activation of the BK_{Ca} channel by LPA in *Xenopus* oocytes expressing rat brain BK_{Ca} channels. (A) The BK_{Ca} channel activation by LPA is concentration-dependent. (a) Representative traces of BK_{Ca} channel current enhancements using different concentrations of LPA (up to 1 μM). (b) The summary histogram shows the effects of various concentrations of LPA on the BK_{Ca} channels. (B) A representative control and LPA-mediated current in an I~V relationship. (a) Currents were recorded at a test potential from -70 to +60 mV, applied from a holding potential of -80 mV. (b) Current-voltage (I~V) relationship of the BK_{Ca} channel in the absence (●) or presence (○) of 100 nM LPA. Voltage pulses with 400-ms duration were applied in 10 mV increments at 3-s intervals from a holding potential of -80 mV. The current peaks, normalized to the peak current evoked by the voltage step to +60 mV in the absence of LPA, were used in the I~V plot. Data represent the mean±S.E.M. (n=4).

voltage step (i.e., a series of voltage pulses of 400 ms duration given in 10 mV increments and 3-s intervals from the holding potential of -80 mV) was used to obtain the I~V curve. In the absence of LPA, voltage pulses greater than 0 mV exhibited slight BK_{Ca} channel currents (Fig. 2Ba, upper traces). The presence of LPA increased the current amplitude over 0 mV compared to the absence of LPA (Fig. 2Ba, lower traces), indicating that LPA activates the BK_{Ca} channel in a voltage-dependent manner (Fig. 2Bb).

We examined the possible changes caused by LPA on BK_{Ca} channel currents after repeated applications of LPA. As shown in Fig. 3A, treatment with 100 nM LPA for 1 min enhanced the BK_{Ca} channel current. Oocytes were then washed with recording buffer for 3 min. When the outward current returned to the basal level, oocytes were re-treated with LPA. The second, third, and fourth BK_{Ca} current responses for LPA were significantly diminished. The BK_{Ca}

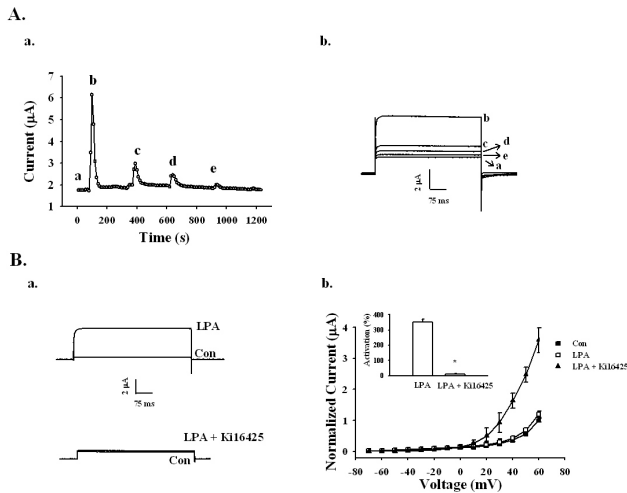


Fig. 3. Effects of repeated treatments of LPA and LPA1/3 receptor antagonist Ki16425 on LPA-mediated BK_{Ca} channel activation. (A-a) Repeated application of 100 nM LPA desensitized the BK_{Ca} channel activation with indicated time interval in representative oocyte (left panel). (A-b) The representative BK_{Ca} channel current traces by repeated LPA treatments were obtained from time-current relationship of “a” (right panel). Outward currents are caused by stepping from -80 mV to $+40$ mV for 400 ms. Data represent the mean \pm S.E.M. ($n=5$). (B-a) Representative traces are from the LPA channel activation in the absence (upper trace) or presence of $10 \mu\text{M}$ Ki16425 for 10 min (lower trace). (B-b) Summary histogram of the LPA effects on BK_{Ca} channel activation (right panel). Data represent the mean \pm S.E.M. ($n=10\sim 12$; $*p < 0.01$).

currents diminished as follows: 410 ± 25 , 137 ± 25 , 34.6 ± 10.3 , $2\pm 1.25\%$, for the first, second, third, and fourth response, respectively ($n=5$, oocytes from two different batches of donors), indicating that repeated treatment of LPA desensitized BK_{Ca} channel activation.

Since the previous report showed that *Xenopus* oocytes express the endogenous LPA1 receptor [21], we examined whether the LPA receptor 1/3 antagonist Ki16425 could inhibit LPA-induced BK_{Ca} channel activation. Oocytes were first incubated with $10 \mu\text{M}$ Ki16425 for 10 min. In the absence of Ki16425, LPA (100 nM) activated the BK_{Ca} channel by $350.52\pm 21.91\%$; however, the presence of Ki16425 LPA (100 nM) activated the BK_{Ca} channel by only $12.50\pm 2.53\%$ (Fig. 3B). Thus, LPA activates the BK_{Ca} channel through LPA receptor activation.

We next investigated the signal transduction pathway for LPA-mediated activation of BK_{Ca} channels. First, we used an active PLC inhibitor, U-73122, and an inactive PLC inhibitor, U-73343 (Fig. 4A). Oocytes expressing the BK_{Ca} channel were incubated with U-73122 ($1 \mu\text{M}$) or U-73343 ($1 \mu\text{M}$) for 20 min. Treatment of LPA with U-73122 significantly attenuated the current from 332.50 ± 26.41 to $41.43\pm 32.95\%$. However, treatment of LPA with U-73343 did not show a significant difference (332.50 ± 26.41 to $315.00\pm 51.05\%$). These results demonstrate that the activation of the BK_{Ca} channel by LPA is also mediated by the PLC pathway.

To study the relationship between the inositol 1,4,5-trisphosphate receptor and BK_{Ca} channel activation by LPA, we used 2-aminoethoxydiphenyl borate (2-APB), which blocks the inositol 1,4,5-trisphosphate (IP₃) receptor. The

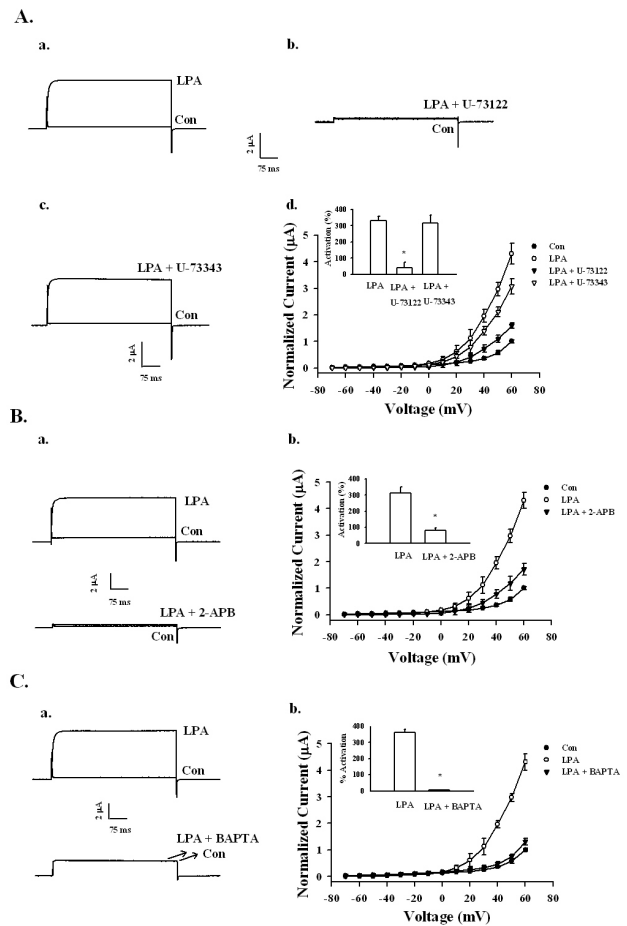


Fig. 4. Signaling pathway of LPA-mediated BK_{Ca} channel activation. (A) Effects of the PLC inhibitor on LPA-mediated BK_{Ca} channel activation. (a~c) A representative trace shows the activation of LPA-(100 nM) mediated currents in *Xenopus* oocytes expressing the BK_{Ca} channel in the absence or presence of inactive U73343 ($1 \mu\text{M}$) or active PLC inhibitor U73122 ($1 \mu\text{M}$). (d) Summary histograms of LPA-mediated activation on control, U-73122, or U-73343-treated groups (right lower panel). Data represent the mean \pm S.E.M. ($n=10\sim 11$; $*p < 0.01$). (B) Effect of IP₃ receptor antagonist 2-APB on LPA-mediated (100 nM) BK_{Ca} channel activation. (a) Representative trace shows the activation of LPA-mediated (100 nM) currents in *Xenopus* oocytes expressing the BK_{Ca} channel in the absence or presence of $100 \mu\text{M}$ 2-APB for 20 min. (b) Summary histograms of LPA-mediated activation on control or $100 \mu\text{M}$ 2-APB (right lower panel). Data represent the mean \pm S.E.M. ($n=12\sim 14$; $*p < 0.05$). (C) Effect of intracellular Ca²⁺ chelator, BAPTA-AM, on LPA-mediated (100 nM) BK_{Ca} channel activation. (a) A representative trace shows the activation of LPA-mediated (100 nM) currents in *Xenopus* oocytes expressing the BK_{Ca} channel in the absence or presence of BAPTA-AM for 3 h. (b) Summary histograms of LPA-mediated activation on control or $100 \mu\text{M}$ BAPTA-AM (right lower panel). Data represent the mean \pm S.E.M. ($n=10\sim 12$; $*p < 0.01$).

oocytes expressing the BK_{Ca} channel were pre-incubated with $100 \mu\text{M}$ 2-APB for 20 min before recording. In the absence of 2-APB, LPA (100 nM) activated the BK_{Ca} channel by $312.63\pm 38.01\%$, whereas in the presence of 2-APB LPA ($1 \mu\text{M}$), the BK_{Ca} channel was activated by only $81.58\pm 13.62\%$ (Fig. 4B). Thus, LPA-mediated activation of the BK_{Ca} channel involves the IP₃ receptor.

Next, we used BAPTA-AM (1,2-bis(*O*-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid), a cell permeable intracellular Ca²⁺ chelator, to discover whether LPA-mediated activation of the BK_{Ca} channel was from elevated levels of free cytosolic Ca²⁺. As shown in Fig. 4C, pretreatment with BAPTA-AM (100 μM, 3 h) on oocytes significantly attenuated LPA-mediated BK_{Ca} channel activation. Thus, the LPA-mediated BK_{Ca} channel current after BAPTA-AM incubation was abolished. Therefore, LPA-mediated enhancement of BK_{Ca} channel currents is dependent on intracellular Ca²⁺.

We also investigated the involvement of the PKC pathway in BK_{Ca} channel activation by LPA. Oocytes expressing the BK_{Ca} channel were incubated with 1.5 μM calphostin, an inhibitor of PKC, for 2 h. Calphostin also significantly attenuated LPA-mediated BK_{Ca} channel activation (Fig. 5A). Thus, LPA-mediated BK_{Ca} channel activation includes the phospholipase C-IP₃ receptor-Ca²⁺-protein kinase C pathways.

The BK_{Ca} channel contains two regulators of K⁺ conductance (RCK) domains in the cytoplasmic domain [3]. We constructed mutant BK_{Ca} channels in the RCK1 domain to decipher whether BK_{Ca} channel activation by LPA involves the RCK1 domain. For this, mutant BK_{Ca} channels with two RCK1s such as M579I and D433A were prepared [22]. In wild-type, LPA (100 nM)-mediated BK_{Ca} channel activa-

tion was 316.5±75.10%. However, in the mutant M579I channel, LPA-mediated BK_{Ca} channel activation was significantly decreased by 30.5±14.12% (Fig. 5Ba). In the mutant D433A channel, LPA-mediated (100 nM) BK_{Ca} channel activation was also significantly decreased by 77.5±38.62% (Fig. 5Ba). The EC₅₀ values for the RCK1 mutants were 121.8±43.0, 134.3±7.9, and 177.2±16.3 nM for wild type, D433A, and M579I, respectively (Fig. 5Ba).

In addition, we constructed six mutant BK_{Ca} channels at the Ca²⁺ bowl, such as D989A, D991A, D992A, D993A, D994A, and D995A [23]. The percentages of activation from 100 nM LPA were 317.50±60.90, 23.60±2.73, 41.20±5.49, 49.16±10.13, 28.14±6.16, 7.05±2.12, and 49.30±16.19% for wild-type, D989A, D991A, D992A, D993A, D994A, and D995A, respectively (n=9). In addition, the EC₅₀ values were 121.80±42.96, 334.22±13.20, 147.54±34.34, 434.53±120.27, 404.29±73.91, 411.30±76.58, and 183.46±3.23 nM for wild-type, D989A, D991A, D992A, D993A, D994A, and D995A, respectively (Fig. 5Bb). These results showed that RCK1 and the Ca²⁺ bowl are involved in LPA-mediated BK_{Ca} channel activation.

DISCUSSION

In the present study, we investigated the signal couplings of LPA on BK_{Ca} channel activation by using a *Xenopus* oocyte gene expression system. Our results revealed three major findings. First, we observed that LPA treatment induced BK_{Ca} channel activation in concentration- and voltage-dependent manners via LPA receptor activation, while repeated treatments with LPA induced rapid desensitization. Second, the presence of PLC inhibitor, IP₃ receptor antagonist, intracellular Ca²⁺ chelator, or PKC inhibitor greatly attenuated the LPA actions. Third, mutations in amino acid residues in the Ca²⁺ bowl and RCK domain greatly attenuated LPA-mediated enhancement of the BK_{Ca} channel currents. Thus, since BK_{Ca} channels play an important role in presynaptic nerve terminals and blood vessel smooth muscle cells, the findings in the present study suggest the possibility that LPA may be a novel BK_{Ca} channel regulator in the nervous and vascular systems via PLC-IP₃-Ca²⁺ signal transduction pathways.

To confirm that the LPA-mediated [Ca²⁺]_i transient is coupled to the activation of BK_{Ca} channels, we first treated oocytes with BAPTA-AM, a membrane permeable Ca²⁺ chelator. As shown in Fig. 4C, BAPTA-AM treatment abolished to 98.5% of the control (p<0.001, compared to BAPTA un-treatment). In addition, we constructed mutant channels in the Ca²⁺ bowl and RCK domain. As shown in Fig. 5B, mutations of several amino acid residues in the Ca²⁺ bowl and RCK domain greatly attenuated LPA-mediated BK_{Ca} channel activation, indicating that extracellular LPA treatment induces the mobilization of cytosolic Ca²⁺ from the endoplasmic reticulum; the increased Ca²⁺ ions bind the Ca²⁺ bowl and RCK domain in order to activate the BK_{Ca} channels.

BK_{Ca} channels are ideal pharmacological targets, since the channel opening increases the attenuation of neuron excitability and smooth muscle relaxation in an existing physiological system. The *in vitro* LPA enhancement of brain BK_{Ca} channel currents also has *in vivo* pharmacological effects. The enhancing effects of LPA on the channel currents may contribute to repolarization of the action potential neurons and vascular smooth muscle cells, while

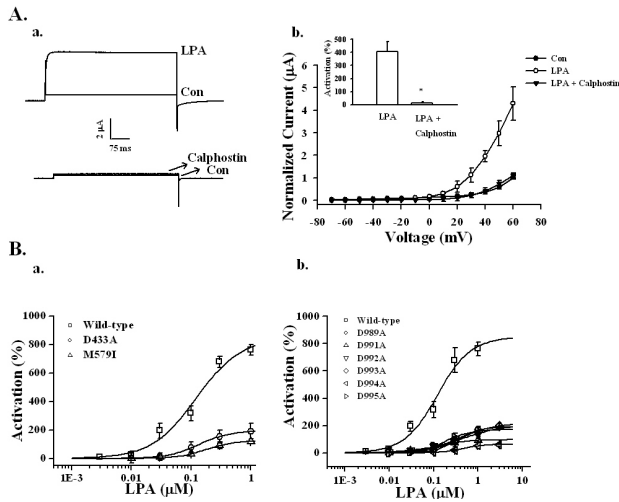


Fig. 5. Effects of PKC inhibitor and mutations in RCK1 and the Ca²⁺ bowl on LPA-mediated BK_{Ca} channel activation. (A-a) Effects of PKC inhibitor on LPA-mediated BK_{Ca} channel activation. A representative trace shows the activation of LPA-mediated (100 nM) currents in *Xenopus* oocytes expressing the BK_{Ca} channel in the absence or presence of 1.5 μM calphostin for 2 h. (A-b) Summary histograms of LPA-mediated BK_{Ca} channel activation in controls or with 1.5 μM calphostin (right lower panel). Data represent the mean±S.E.M. (n=10~12; *p<0.01). (B) Effect of mutations on the RCK1 and Ca²⁺ bowl domains in LPA-mediated BK_{Ca} channel activation. (B-a) The oocytes expressing wild-type or mutant BK_{Ca} channels in the RCK domain such as D433A or M579I were treated with LPA by a bathing application for 60 s. Mutations in the RCK domain attenuated the LPA concentration-responses (left panel). (B-b) The oocytes expressing wild-type or mutant BK_{Ca} channels in the Ca²⁺ bowl were treated with LPA by a bathing application for 60 s. Mutations in the Ca²⁺ bowl also attenuated LPA concentration-responses (right panel). Data represent the mean±S.E.M. (n=6).

shortening the action potential duration. Thus, activating BK_{Ca} channels by LPA might be used to reduce over-excitability of the nervous system or to down-regulate hyperactivity of vascular smooth muscle cells. This study also raises the possibility that LPA participates in the regulation of synaptic transmission in nerve terminals and vascular systems.

On the other hand, recent report showed that LPA is also related with pain induction in spinal cord, since LPA synthesized in the spinal dorsal horn after nerve injury [24]. LPA-mediated intracellular [Ca²⁺]_i increase not only stimulates microglia to induce the BDNF synthesis through activation of P₂X₄ receptors, but also activates activation of microglial BK_{Ca} channels [25]. BDNF secreted from microglia in turn further enhance the activities of BK channels [19,26]. Thus, it seems that the physiological or pharmacological roles of LPA-mediated BK channel activations might be dependent on cell types or organs. Further investigations into the potential clinical applications of LPA on the nervous and vascular systems are required.

In conclusion, we found that activation of G_{α_{q11}} protein-coupled LPA receptor by LPA is coupled to BK_{Ca} channel activation and that Ca²⁺ bowl and RCK domain of BK_{Ca} channel also play important roles in LPA-mediated BK_{Ca} channel activation using site-directed mutagenesis method. The present findings may provide a molecular basis for LPA effects on the nervous and vascular systems.

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REFERENCES

- Ghatta S, Nimmagadda D, Xu X, O'Rourke ST. Large-conductance, calcium-activated potassium channels: structural and functional implications. *Pharmacol Ther.* 2006;110:103-116.
- Salkoff L, Butler A, Ferreira G, Santi C, Wei A. High-conductance potassium channels of the SLO family. *Nat Rev Neurosci.* 2006;7:921-931.
- Yuan P, Leonetti MD, Pico AR, Hsiung Y, MacKinnon R. Structure of the human BK channel Ca²⁺-activation apparatus at 3.0 Å resolution. *Science.* 2010;329:182-186.
- Weiger TM, Hermann A, Levitan IB. Modulation of calcium-activated potassium channels. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol.* 2002;188:79-87.
- Fahanik-Babaei J, Eliassi A, Saghiri R. How many types of large conductance Ca²⁺-activated potassium channels exist in brain mitochondrial inner membrane: evidence for a new mitochondrial large conductance Ca²⁺-activated potassium channel in brain mitochondria. *Neuroscience.* 2011;199:125-132.
- Jiang Y, Pico A, Cadene M, Chait BT, MacKinnon R. Structure of the RCK domain from the E. coli K⁺ channel and demonstration of its presence in the human BK channel. *Neuron.* 2001;29:593-601.
- Lee US, Cui J. BK channel activation: structural and functional insights. *Trends Neurosci.* 2010;33:415-423.
- Niu X, Qian X, Magleby KL. Linker-gating ring complex as passive spring and Ca²⁺-dependent machine for a voltage- and Ca²⁺-activated potassium channel. *Neuron.* 2004;42:745-756.
- Tigyi G. Aiming drug discovery at lysophosphatidic acid targets. *Br J Pharmacol.* 2010;161:241-270.
- Contos JJ, Ishii I, Chun J. Lysophosphatidic acid receptors. *Mol Pharmacol.* 2000;58:1188-1196.
- Moolenaar WH, Kranenburg O, Postma FR, Zondag GC. Lysophosphatidic acid: G-protein signalling and cellular responses. *Curr Opin Cell Biol.* 1997;9:168-173.
- Dyer D, Tigyi G, Miledi R. The effect of active serum albumin on PC12 cells: I. Neurite retraction and activation of the phosphoinositide second messenger system. *Brain Res Mol Brain Res.* 1992;14:293-301.
- Chettibi S, Lawrence AJ, Stevenson RD, Young JD. Effect of lysophosphatidic acid on motility, polarisation and metabolic burst of human neutrophils. *FEMS Immunol Med Microbiol.* 1994;8:271-281.
- Ha TS, Lim HH, Lee GE, Kim YC, Park CS. Electrophysiological characterization of benzofuroindole-induced potentiation of large-conductance Ca²⁺-activated K⁺ channels. *Mol Pharmacol.* 2006;69:1007-1014.
- Choi S, Rho SH, Jung SY, Kim SC, Park CS, Nah SY. A novel activation of Ca²⁺-activated Cl⁻ channel in *Xenopus* oocytes by Ginseng saponins: evidence for the involvement of phospholipase C and intracellular Ca²⁺ mobilization. *Br J Pharmacol.* 2001;132:641-648.
- Lee JH, Lee BH, Choi SH, Yoon IS, Pyo MK, Shin TJ, Choi WS, Lim Y, Rhim H, Won KH, Lim YW, Choe H, Kim DH, Kim YI, Nah SY. Ginsenoside Rg3 inhibits human Kv1.4 channel currents by interacting with the Lys531 residue. *Mol Pharmacol.* 2008;73:619-626.
- Lu L, Montrose-Rafizadeh M, Guggino WB. Ca²⁺-activated K⁺ channels from rabbit kidney medullary thick ascending limb cells expressed in *Xenopus* oocytes. *J Biol Chem.* 1990;265:16190-16194.
- Candia S, Garcia ML, Latorre R. Mode of action of iberiotoxin, a potent blocker of the large conductance Ca²⁺-activated K⁺ channel. *Biophys J.* 1992;63:583-590.
- Langer P, Gründer S, Rüscher A. Expression of Ca²⁺-activated BK channel mRNA and its splice variants in the rat cochlea. *J Comp Neurol.* 2003;455:198-209.
- Kaczorowski GJ, Knaus HG, Leonard RJ, McManus OB, Garcia ML. High-conductance calcium-activated potassium channels; structure, pharmacology, and function. *J Bioenerg Biomembr.* 1996;28:255-267.
- Kimura Y, Schmitt A, Fukushima N, Ishii I, Kimura H, Nebreda AR, Chun J. Two novel *Xenopus* homologs of mammalian LP(A1)/EDG-2 function as lysophosphatidic acid receptors in *Xenopus* oocytes and mammalian cells. *J Biol Chem.* 2001;276:15208-15215.
- Yusifov T, Savalli N, Gandhi CS, Ottolia M, Olcese R. The RCK2 domain of the human BKCa channel is a calcium sensor. *Proc Natl Acad Sci U S A.* 2008;105:376-381.
- Schreiber M, Salkoff L. A novel calcium-sensing domain in the BK channel. *Biophys J.* 1997;73:1355-1363.
- Ma L, Uchida H, Nagai J, Inoue M, Aoki J, Ueda H. Evidence for de novo synthesis of lysophosphatidic acid in the spinal cord through phospholipase A2 and autotaxin in nerve injury-induced neuropathic pain. *J Pharmacol Exp Ther.* 2010;333:540-546.
- Hayashi Y, Kawaji K, Sun L, Zhang X, Koyano K, Yokoyama T, Kohsaka S, Inoue K, Nakanishi H. Microglial Ca²⁺-activated K⁺ channels are possible molecular targets for the analgesic effects of S-ketamine on neuropathic pain. *J Neurosci.* 2011;31:17370-17382.
- Gao Y, Yang Y, Guan Q, Pang X, Zhang H, Zeng D. IL-1β modulate the Ca²⁺-activated big-conductance K channels (BK) via reactive oxygen species in cultured rat aorta smooth muscle cells. *Mol Cell Biochem.* 2010;338:59-68.