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SIGNALING AND CELL PHYSIOLOGY

The GPR55 agonist lysophosphatidylinositol acts as an intracellular messenger and bidirectionally modulates Ca^{2+}-activated large-conductance K^+ channels in endothelial cells

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Abstract Lysophospholipids are known to serve as intra- and extracellular messengers affecting many physiological processes. Lysophosphatidylinositol (LPI), which is produced in endothelial cells, acts as an endogenous agonist of the orphan receptor, G protein-coupled receptor 55 (GPR55). Stimulation of GPR55 by LPI evokes an intracellular Ca^{2+} rise in several cell types including endothelial cells. In this study, we investigated additional direct, receptor-independent effects of LPI on endothelial large-conductance Ca^{2+} and voltage-gated potassium (BK_{Ca}) channels. Electrophysiological experiments in the inside-out configuration revealed that LPI directly affects the BK_{Ca} channel gating properties. This effect of LPI strictly depended on the presence of Ca^{2+} and was concentration-dependent, reversible, and dual in nature. The modulating effects of LPI on endothelial BK_{Ca} channels correlated with their initial open probability (Po): stimulation at low Po (<0.3) and inhibition at high Po levels (>0.3). In the whole-cell configuration, LPI in the pipette facilitated membrane hyperpolarization in response to low (0.1–2 μM) histamine concentrations. In contrast, LPI counteracted membrane hyperpolarization in response to supramaximal cell stimulation with histamine. These results highlight a novel receptor-independent and direct bidirectional modulation of BK_{Ca} channels by LPI on endothelial cells. We conclude that LPI via this mechanism serves as an important modulator of endothelial electrical responses to cell stimulation.

Keywords BK_{Ca} channel · Cytosolic free Ca^{2+} elevation · Endothelial cells · Hyperpolarization · Membrane potential · Lipid mediators · Lysophosphatidylinositol

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Introduction

Lysophosphatidylinositol (LPI) belongs to the class of lysophospholipids and is generated by phosphatidylinositol hydrolysis via the action of the Ca^{2+}-dependent phospholipase A2 [2, 27] and Ca^{2+}-independent phospholipase A1 [27, 45]. LPI has been found to contribute to many physiological and pathophysiological processes including reproduction, angiogenesis, cell proliferation, apoptosis, inflammation, cardiovascular, and autoimmune diseases [6, 10, 25]. Because of the discovery that LPI serves as a putative endogenous agonist of the orphan receptor G protein-coupled receptor 55 (GPR55) [24, 32, 33, 42], which leads to mobilization of intracellular Ca^{2+}, LPI recently received particular attention. Moreover, LPI was shown to stimulate non-selective cation channels and inhibit the Na^+/K^+ ATPase [4] GPR55-independently pointing to LPI as a putative vascular mediator.

An increase in intracellular free Ca^{2+} concentration represents a hallmark in endothelial cell physiology. Subsequently to the rise in cytosolic Ca^{2+}, Ca^{2+}-dependent K^+ channels (K_{Ca}) become activated [8, 9, 20] that they play a pivotal role in the regulation of vascular tone. In vascular smooth muscle cells, stimulation of large-

conductance Ca^{2+} and voltage-gated potassium (BK_{Ca}) channels counteracts depolarization during myogenic tone development, thus, limiting voltage-dependent Ca^{2+} entry and counteracting vasoconstriction. In endothelial cells, the stimulation of K_{Ca} contributes to vasodilatation by membrane hyperpolarization that increases the driving force for Ca^{2+} entry through non-voltage-gated Ca^{2+} channels that subsequently yields Ca^{2+}-dependent formation of vasodilators, such like nitric oxide [18, 19, 30] or endothelial-derived hyperpolarizing factors [5, 16].

So far, stimulatory effects of LPI on BK_{Ca} and intermediate conductance K_{Ca} channels were reported to depend on the ability of LPI to mobilize internal Ca^{2+} [12, 38, 43]. However, recently several endogenous phospholipids, such like phosphatidylinositol 4, 5-bisphosphate or sphingosine-1-phosphate, have been shown to activate BK_{Ca} [26, 40] as well as TRPC channels [14] directly. Thus, the actual effects of LPI on vascular BK_{Ca} channels ought to be investigated in order to gain the understanding of the physiological potential of this putative inter- and intravascular mediator.

Accordingly, in order to explore potential direct effects of LPI on endothelial BK_{Ca} channels that lack the regulatory β-subunit [34], the effects of LPI were explored in excised membrane patches from human endothelial cells. We report herein that LPI directly modifies BK_{Ca} channel activity in a dual manner by either potentiating or inhibiting native BK_{Ca} channels at lower and higher basal activity of the channel, respectively. Altogether, our data indicate that LPI, besides its activity on GPR55, also exhibits receptor-independent effects, thus, pointing to LPI as a versatile messenger in the vasculature.

Materials and methods

Cell culture

The human umbilical vein-derived endothelial cell line, EA.hy926 [11], at passage >45, was grown in DMEM containing 10% FCS and 1% HAT (5 mM hypoxanthine, 20 μM aminopterin, 0.8 mM thymidine) and were maintained in an incubator at 37°C in 5% CO_2 atmosphere. For experiments, cells were plated on glass coverslips.

Patch clamp recordings

Single-channel recordings were obtained from excised inside-out membrane patches in symmetrical solutions using the patch clamp technique. The pipettes were filled with (in millimolar) 140 KCl, 10 HEPES, 1 $MgCl_2$, 5 EGTA, 4,931 $CaCl_2$ with pH 7.2 by adding KOH (i.e., 10 μM free Ca^{2+}, calculated by CaBuf from G. Droogmans, Leuven, Belgium; ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip). Cells were perfused with a bath solution containing (in millimolar) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, 2.4 $CaCl_2$. Following gigaseal formation, bath solution was switched to the following (in millimolar) 140 KCl, 10 HEPES, 1 $MgCl_2$, 5 EGTA, and a desired free Ca^{2+} concentration which was adjusted by adding different amounts of $CaCl_2$ calculated by the program CaBuf; pH was adjusted to 7.1 by adding KOH.

For whole-cell recordings, the pipette solution contained (in millimolar) 100 K-aspartate, 40 KCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA, and a free [Ca^{2+}] was adjusted to 100 nM by adding 1,924 $CaCl_2$ calculated by the program CaBuf. Recordings were performed in high Na^+ solution stated above. Patch pipettes were pulled from glass capillaries using a Narishige puller (Narishige Co. Ltd, Tokyo, Japan), fire-polished, and had a resistance of 3–5 MΩ for whole-cell recordings and 5–7 MΩ for single-channel recordings. Currents were recorded using a patch clamp amplifier (EPC7, List Electronics, Darmstadt, Germany) at a bandwidth of 3 kHz. The signals obtained were low pass filtered at 1 kHz using an eight-pole Bessel filter (Frequency Devices) and digitized with a sample rate of 10 kHz using a Digidata 1200A A/D converter (Axon Instruments, Foster City, CA, USA). Data collection and analysis were performed using Clampex and Clampfit software of pClamp (V9.0, Axon Instruments). Single-channel activity was obtained from >20 s of continuous recording under each experimental condition. The mean open time (to) in multichannel patches was calculated from the relationship $to = \frac{NPo \times T}{\#o}$, where *#o* is the number of openings during a given time period of observation (*T*) [40].

Statistics

Analysis of variance (ANOVA) was performed, and statistical significance was evaluated using Scheffé's post hoc *F* test of the Prism 5 software for Windows (GraphPad Software, Avenida de la Playa, CA USA). Level of significance was defined as $P<0.05$.

Results

LPI directly affects the BK_{Ca} channel activity by shortening the channel closed time

To investigate whether LPI has a direct effect on BK_{Ca} channels, the action of LPI on BK_{Ca} channel activity was investigated in the inside-out configuration in the presence of physiological levels of Ca^{2+} and Mg^{2+}, the two metal ligands that affect gating of BK_{Ca} channels. Figure 1a illustrates BK_{Ca} single-channel activities recorded in ex-

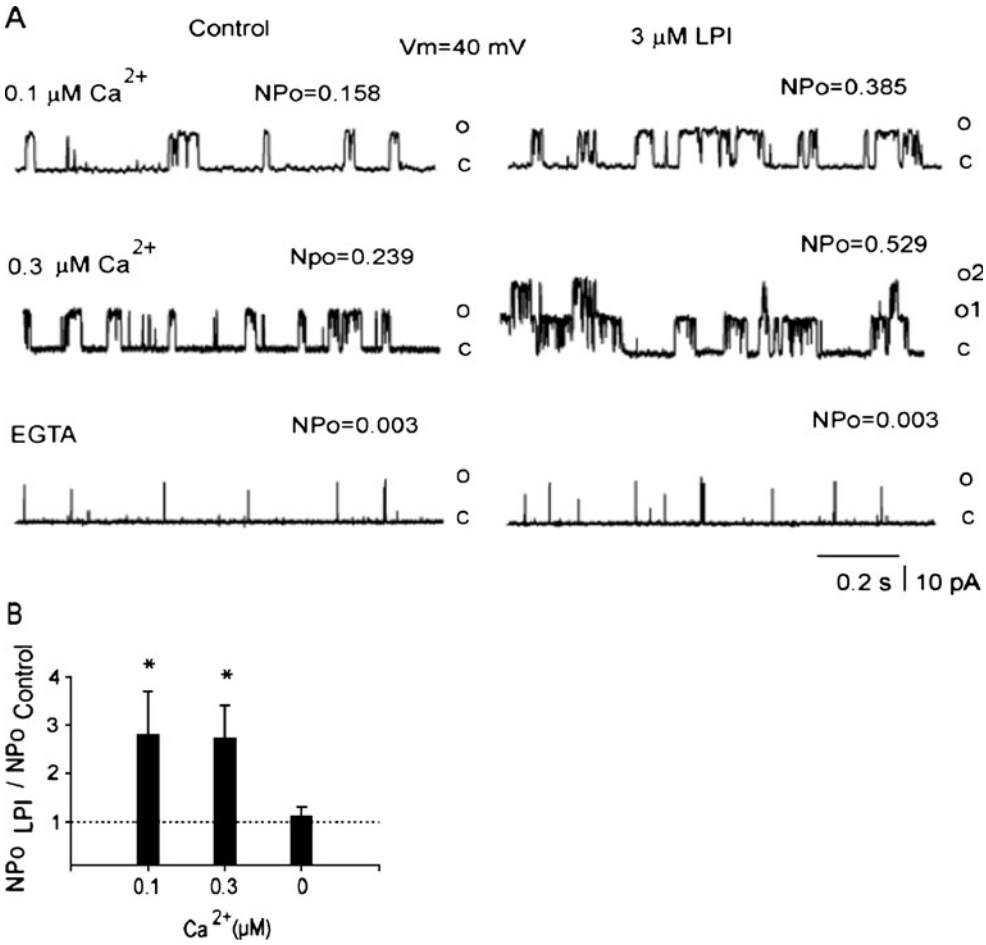

Fig. 1 Ca^{2+} is required for the direct modulation of BK channel activity by LPI. **a** Representative single-channel recordings in inside-out patch (patch potential=+40 mV) exposed to 0.1 (*top panels*) and 0.3 μM (*middle panels*) Ca^{2+} prior (control) and after addition of 3 μM LPI to the cytosolic side of the patch. Channel openings are shown as upward deflections (*c*, *closed*, *o*, *open*). In the absence of Ca^{2+}, LPI has no effect on BK_{Ca} channel activity (*lower panels*). **b** Summary data for the effect of 3 μM LPI on BK_{Ca} channel activity in the absence (0 mM Ca^{2+}; n=6) and presence of 0.1 (n=10) and 0.3 μM (n=21) free Ca^{2+} in the bath. *p< 0.05 vs. basal NPo in the absence of LPI.

cised patches in the presence of 0.1 (top panel) and 0.3 μM free Ca^{2+} (middle panel), as well as in the nominal absence of bath Ca^{2+} (lower panel), prior (left panels) and after addition of 3 μM LPI (right panels) to the bath at a holding potential of +40 mV. In the presence of bath Ca^{2+}, LPI increased the NPo values of given BK_{Ca} channel of the patch by ~2.8-fold, while LPI was ineffective to stimulate BK_{Ca} channels in the absence of free Ca^{2+} in the bath (Fig. 1b). Because the stimulatory effects of LPI were observed after patch excision in Ca^{2+}-containing solution, it is tempting to suggest that the action of LPI on BK_{Ca} channels occurs due to a direct interaction of LPI with the channels or some closely associated entities but not via the generation of a cytosolic second messenger.

The increase in NPo upon LPI addition was sustained (Fig. 2a) and reversible, following a 15-min washout (Fig. 2b). In addition, the stimulatory effect of LPI on BK_{Ca} channels was concentration-dependent (Fig. 2c, d). Due to a loss of the gigaseal, we were not able to explore the effect of LPI on BK_{Ca} channel activities at concentrations higher than 10 μM LPI. Notably, the LPI concentrations tested are within the range that are described to be found in humans under physiological/pathological conditions [10, 44] and are well below the critical micellar concentration (~75 μM; [13]).

In order to characterize the effect of LPI in more detail, experiments with patches with only one active channel were performed. The existence of just one active BK_{Ca} channel in the respective patches was determined by applying 10 μM Ca^{2+} solution at the end of the experiments at a holding potential of +80 mV. From these experiments, we found that LPI increased the channel Po, while the single-channel amplitude remained unchanged (Fig. 3a). Moreover, the mean open time was moderately increased by LPI (Fig. 3b), while LPI strongly attenuated the mean closed time by ~77% reflecting an increase in opening frequency (Fig. 3c), thus indicating that the increase in Po by LPI is mainly due to a shortening of the closed time rather than a prolongation of the channel open time.

LPI dually modulates BK_{Ca} channels depending on the basal channel activity

Because Ca^{2+} is required for the stimulatory action of LPI, LPI may act via an increase in the apparent Ca^{2+} sensitivity of the BK_{Ca} channel. Thus, the effect of LPI on BK_{Ca}

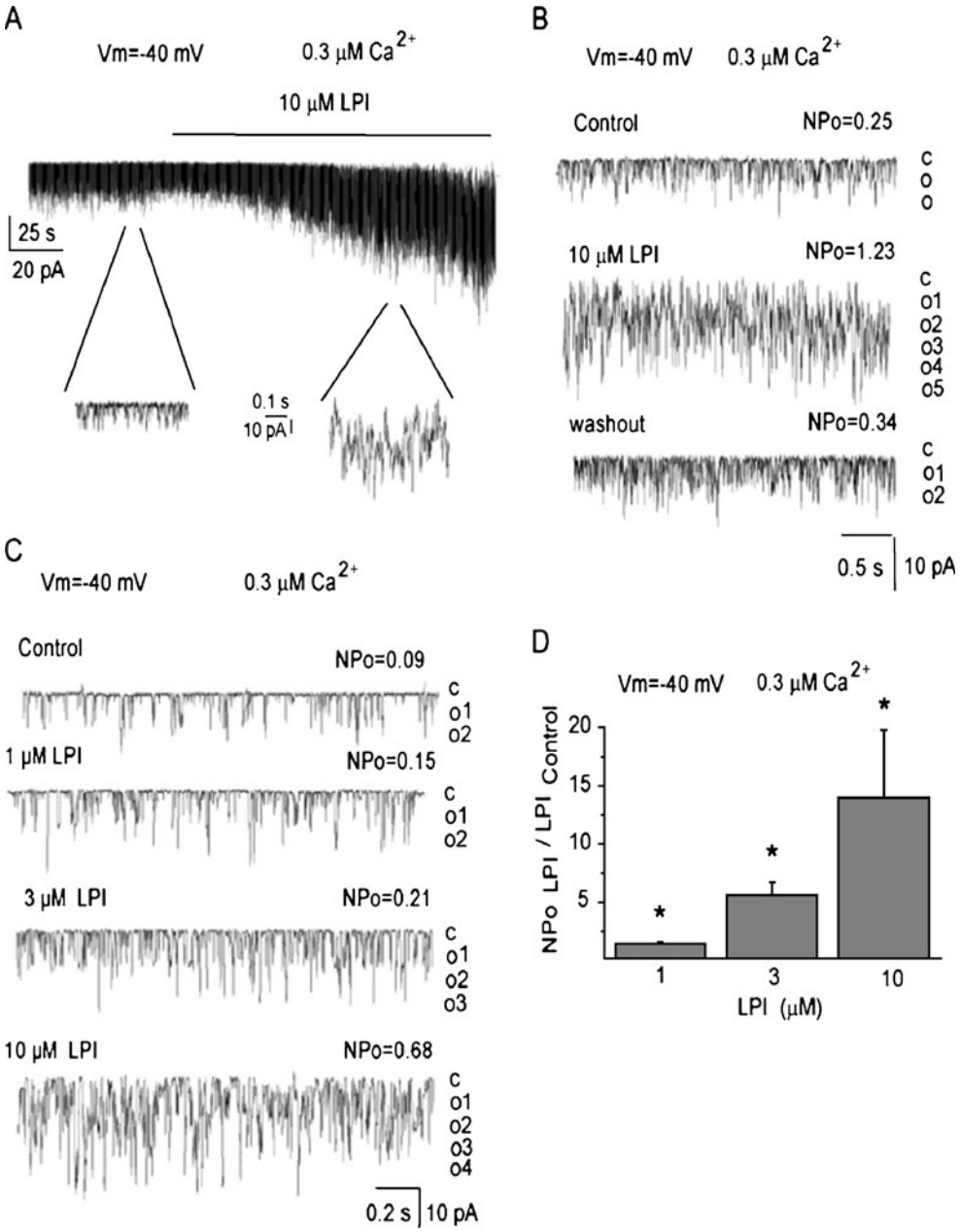

Fig. 2 The LPI effect develops slowly, is sustained, reversible, and occurs in a concentration-dependent manner. **a** Representative single-channel recording in inside-out patch exposed to 10 μM LPI in the presence of 0.3 μM bath Ca^{2+} holding potential (Vm)=−40 mV. Channel openings are shown as downward deflections. **b** The reversibility of the effect of LPI effect is demonstrated by this representative single-channel recording in inside-out patch exposed to 0.3 μM Ca^{2+} prior (*top*, control), during (*middle*), and 15 min after exposure to 10 mM LPI (Vm=−40 mV). **c** Representative single-channel recording in inside-out patch exposed to 0.3 μM Ca^{2+} showing concentration-dependent activation of BK_{Ca} channel activity in response to bath application of 1, 3, and 10 μM LPI. Channel openings are shown as downward deflections (*c*, *closed*, *o*, *open state*). **d** Statistical representation of the effect of 1 (n=6), 3 (n=7), and 10 μM (n=4) LPI on BK_{Ca} channels in the presence of 0.3 μM Ca^{2+} at Vm=−40 mV. *p< 0.05 vs. basal NPo in the absence of LPI

channel activity was studied in the presence of various Ca^{2+} concentrations in the bath. Interestingly, depending on the bath Ca^{2+} concentration, LPI exhibited opposite effects on the activity of the BK_{Ca} channels. In particular, under conditions of low Ca^{2+} in the bath solution (i.e., 0.1 and 0.3 μM free Ca^{2+}), which were associated with basal Po lower than 0.3, the addition of 3 μM LPI augmented BK_{Ca} channel activity (Fig. 4a). In contrast, at high Ca^{2+} concentrations in the bath (i.e., 1 and 10 μM) that yielded elevated basal BK_{Ca} activity, LPI decreased the activity of the BK_{Ca} channel (Fig. 4a). This inhibitory effect of LPI was observed both in multichannel patches (Fig. 4b) and patches containing one active channel (Fig. 4c), and occurred in a concentration-dependent manner. This concentration-dependent inhibitory effect of LPI was in a similar range than that for the activation properties of LPI (Fig. 4d). Dwell time analysis of experiments performed in single-channel patches revealed that upon LPI exposure, the mean open time slightly decreased by 16±10% (Fig. 4e), while the mean closed time strongly increased by 168±82% (Fig. 4f). The decrease in (N)Po upon LPI under conditions of high bath Ca^{2+} is due to a pronounced increase in the mean closed time (a decrease in the frequency of openings) and a small decrease in the mean open time.

We hypothesized that these striking differences of the response of BK_{Ca} channel activity to LPI is due to the variability of the basal Po values. If this is the case, the basal Po levels should correlate with the degree of changes

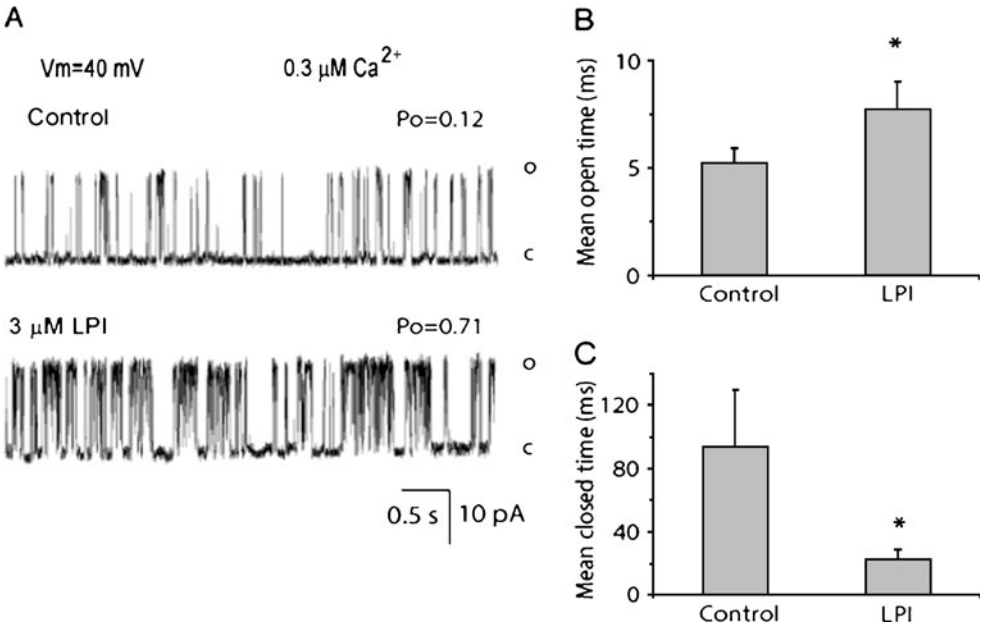

Fig. 3 LPI-evoked increase in BK_{Ca} channel activity is mainly due to a marked decrease in mean closed time. **a** Representative single-channel recording in inside-out patch containing one active channel exposed to 0.3 μM Ca^{2+} prior (control) and during exposure to 3 μM LPI at the cytosolic side of the patch (Vm=40 mV). Channel openings are shown as upward deflections (*c*, *closed*, *o*, *open states*). **b** The mean open time of the BK_{Ca} prior (control) and after addition of 3 μM LPI (n=12). **c** The mean closed time (frequency of openings) of the BK_{Ca} prior (control) and after addition of LPI (n=12). *p<0.05 vs. control

in BK_{Ca} channel activities caused by LPI. Plotting NPo responses against the basal Po values of BK_{Ca} channels in patches exposed to various bath Ca^{2+} concentration at a holding potential of +40 mV (Fig. 4g) revealed a clear inverse correlation between these two parameters (r^2= 0.77). This finding indicates that the overall effect of LPI indeed depends on the basal Po level and thus on the basal activities of BK_{Ca} channels. Particularly, these data specify that for BK_{Ca} channels with a basal Po higher than 0.3 LPI exhibits an inhibitory action, while at a lower basal activity of the channels, LPI yields a stimulation of BK_{Ca} channel activity.

Because dual sensitivities to Ca^{2+} and transmembrane voltage are key features of BK_{Ca} channels, we next studied whether LPI affects the voltage-dependency of BK_{Ca} channels. In single-channel recordings, the stepwise elevation of the holding potential from 20 to 80 mV considerably increased the BK_{Ca} channel activity (Fig. 5a, left panels). This increase was further potentiated by 3 μM LPI at every potential tested (Fig. 5a, right panels). In terms of channel-opening characteristics at different voltages, LPI slightly affected the mean open time of the BK_{Ca} channels (Fig. 5c), while the channel's mean closed time was strongly reduced by LPI (Fig. 5d). These experiments suggest that voltage dependency of the BK_{Ca} channels is barely affected by LPI.

Furthermore, weak voltage-dependent effect of LPI was observed in experiments depicted in Fig. 6 and was performed in the presence of a given Ca^{2+} concentration of 10 μM. At negative voltages (i.e., −60 and −40 mV), when the channel activity was low (NPo=0.173 at −60 mV and 0.282 at −40 mV, Fig. 6a), LPI equally increased NPo values (2.7- and 2.5-fold, respectively) (Fig. 6b, c). However, at positive voltages (i.e., +40 and Vm= +60 mV) that were associated with high basal activity of the channel (NPo levels of 1.23 at +40 mV and 1.27 at +60 mV), LPI equally decreased the channel activity by ~29% and 28%, respectively (Fig. 6b, c). Altogether, these data indicate that LPI modifies BK_{Ca} channel activity mainly through modulating Ca^{2+} sensitivity of the BK_{Ca} channels, and the effect of LPI strictly depends on the actual state of the activity of these channels.

Intracellular LPI dually modifies endothelial electrical responses to histamine

Because LPI dually modifies BK_{Ca} channel activities in excised patches in a Ca^{2+}-dependent manner, we next explored the physiological relevance of these findings by testing the impact of physiologically reported LPI concentrations (i.e., from 0.1 to 10 μM; [44]) on endothelial electrical responses to histamine in conventional whole-cell recordings. Because in the endothelial cell type used for this study, histamine-induced hyperpolarization is partially underpinned by an activation of BK_{Ca} channels [15], the effect of 0.1 or 1.0 μM LPI in the patch pipette on membrane hyperpolarization in response to various moderate histamine concentrations was tested. Histamine evoked membrane hyperpolarization of endothelial cells in a concentration-dependent manner (Fig. 7a, d). Under control conditions (no LPI in pipette) consecutive applications of 0.1, 0.5, and 2.0 μM histamine produced hyperpolarizing responses of 10.5±2.1 mV (n=19), 19.8±2.3 mV (n=16), and 24.8±4.4 (n=7) from the mean resting membrane potential of −33.8±1.7 mV (n=19) (Fig. 7d). In the presence of 0.1 or 1.0 μM LPI in the pipette, the resting membrane potential was not affected compared with control

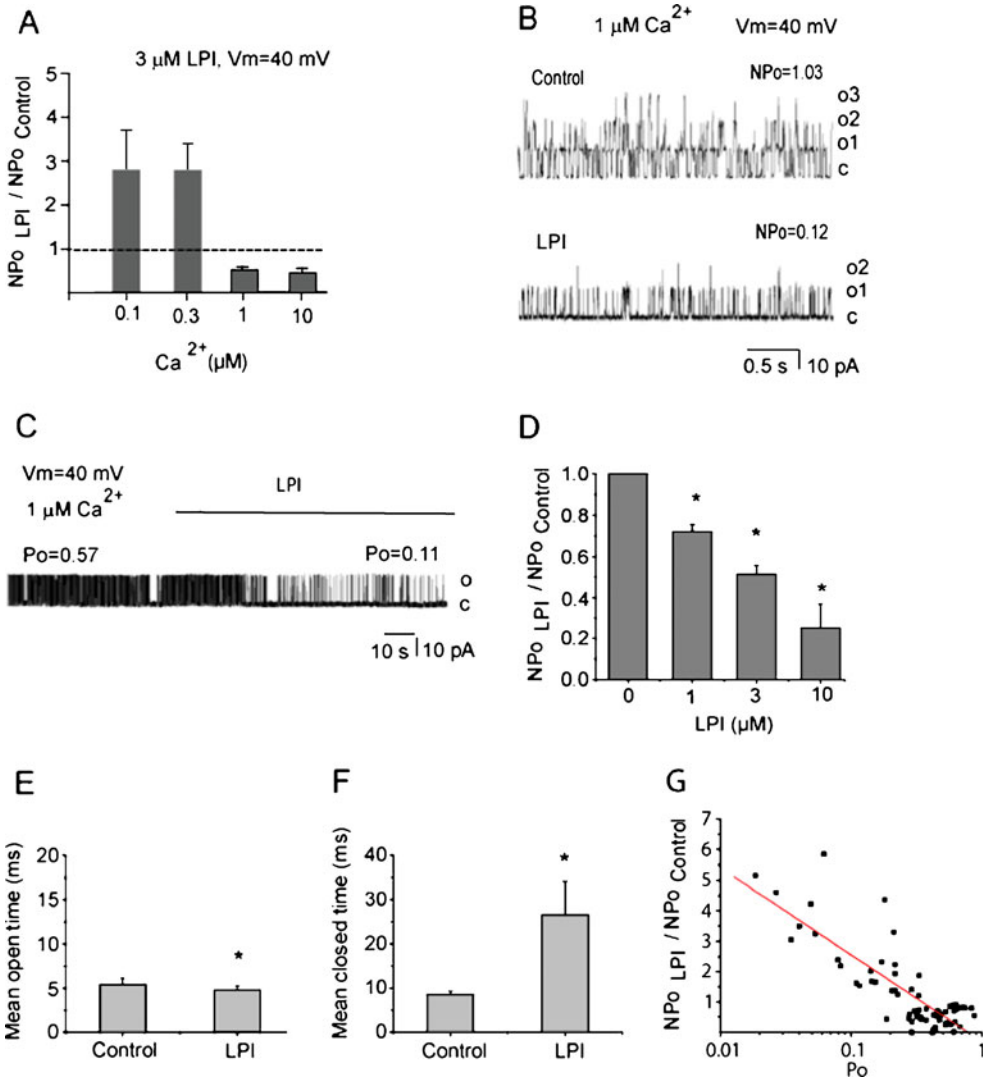

Fig. 4 The dual effect of LPI on BK_{Ca} channel activity. **a** Effect of 3 μM LPI on BK_{Ca} channel activity at different bath Ca^{2+} concentrations that correspond to basal (0.1 μM; n=10), moderately (0.3 μM; n=21), strong (1 μM; n=20), and maximally (10 μM; n=20) elevated cytosolic Ca^{2+} levels. *p< 0.05 vs. basal NPo in the absence of LPI. **b** Representative single-channel recordings from multichannel patch exposed to 1 μM Ca^{2+} at Vm=+40 mV with high basal NPo showing inhibitory effect of 3 μM LPI on BK_{Ca} channel activity. Channel openings are shown as upward deflections (*c*, *closed*; *o*, *open state*). *Right panel*: statistical representation of Po values before (control) and after addition of 3 μM LPI in patches exposed to 1 μM Ca^{2+} at Vm=±40 mV and responded by a decrease in BK_{Ca} channel activity. **c** Representative single-channel recording in inside-out patch containing one active channel with the basal Po=0.57 showing inhibitory effect of 3 μM LPI on BK_{Ca} channel activity. The patch was exposed to 1 μM Ca^{2+} at Vm=+40 mV. **d** Summary data of the concentration-dependency of the inhibitory effect of LPI on BK_{Ca} channel activity in patches exposed to 1 μM Ca^{2+} at Vm=+40 mV (n=6–21) *p< 0.05 vs. control. The respective mean open time (**e**; n= 6, *p< 0.05) and the mean closed time (**f**; n=6, *p< 0.05) of the BK_{Ca} channel prior (control) and after addition of 3 μM LPI (Vm=+40 to +60 mV). Data collected from patches exposed to 1 and 10 μM Ca^{2+} were pooled. **g** Correlation between the LPI-evoked alterations in BK_{Ca} channel activity (expressed as the ratio of NPo values in the presence and absence of 3 μM LPI) and basal Po values at Vm=+40 mV. Data points were obtained in the presence of 0.1, 0.3, 1, and 10 μM Ca^{2+}

conditions (−32.0±2.0 mV (n=12) and −32.4±1.6 (n=8), respectively). However, when 0.1 and 1.0 μM LPI was present in the pipette, the amplitude of cell hyperpolarization in response to low histamine concentrations increased (Fig. 7b–d). To provide a link between increased endothelial hyperpolarization to low histamine concentrations in the presence of intacellular LPI and stimulatory effect of LPI on BK_{Ca} channels, paired experiments were conducted in the presence and absence of iberiotoxin, a selective inhibitor of BK_{Ca} channels. In the absence of intrapipette LPI, iberiotoxin (100 nM) slightly but significantly (p< 0.05) reduced the peak amplitude to 0.5 and 2 μM histamine from 23.5±3.3 to 20.9±2.7 (n=8) and from 28.5±3.0 to 25.4±3.3 mV (n=8), respectively. In the

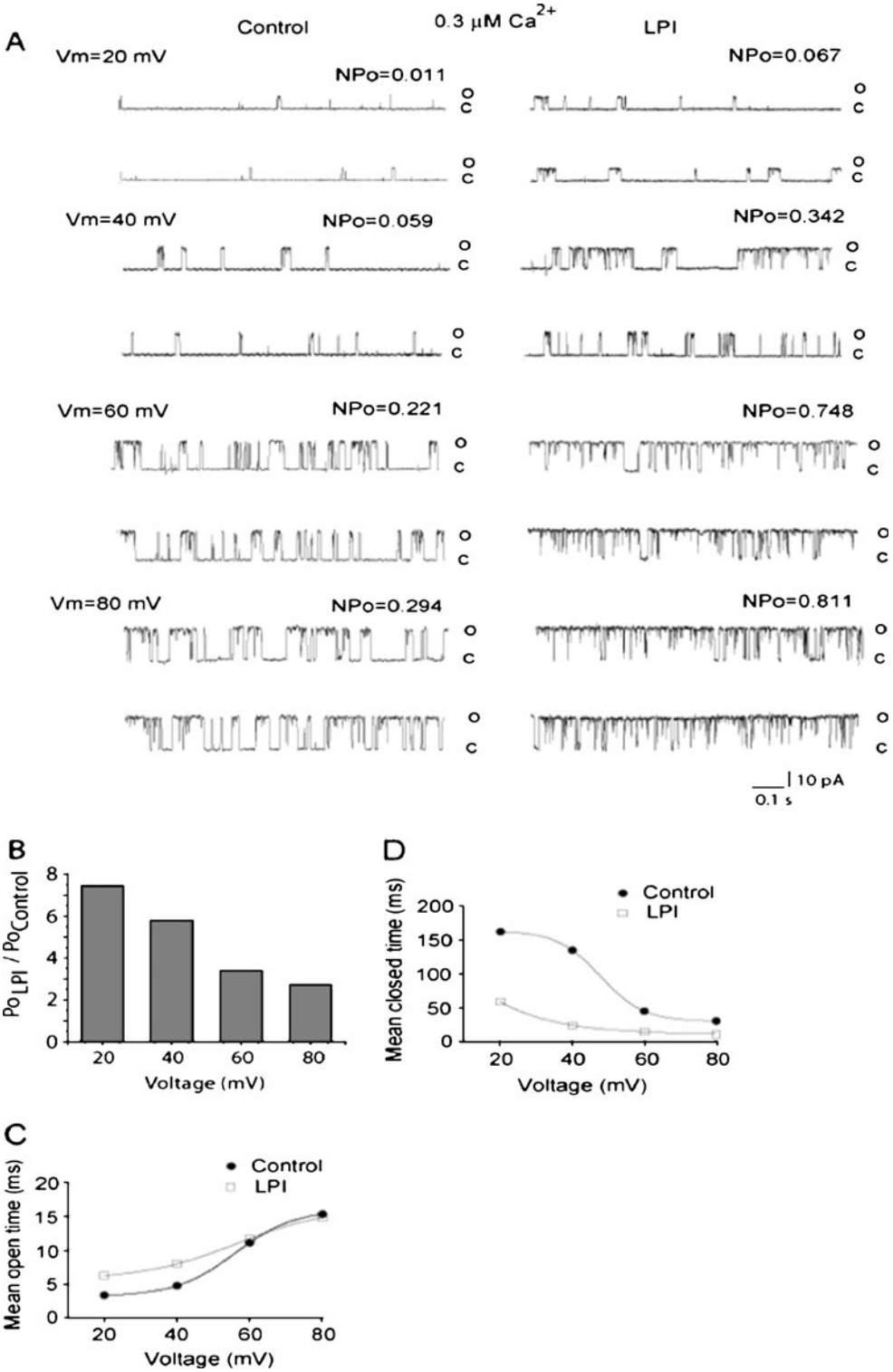

Fig. 5 Effect of LPI on voltage-sensitivity of BK_{Ca} channel. **a** The activity of BK_{Ca} channels prior (*left*) and after (*right*) bath application of 3 μM LPI. The patch was exposed to 1 μM Ca^{2+}. Unitary currents were recorded at different membrane potentials as indicated. Upward deflections are the opening events of the channel (*c*, *closed*, *o*, *open* state). **b** Correlation of the effect of 3 μM LPI on the channels Po expressed as percent of control with the actual holding potential. The relationship between the mean open time (**c**) and mean closed time (**d**) of the channel with the actual holding potential in the absence (control) and presence of LPI (3 μM). Data presented are representative data and experiments were repeated four times with different patches that provided similar results

presence of intrapipette LPI (1 μM), iberiotoxin (100 nM) slightly but significantly ($p<0.05$) reduced the peak amplitude to 0.5 and 2 μM histamine (Fig. 7e, f) from 29.4±2.8 to 24.9±2.9 mV ($n=7$) and from 34.9±2.2 to 29.4±1.5 mV ($n=11$), respectively. Remarkably, iberiotoxin failed to completely inhibit the stimulatory effect of LPI on endothelial hyperpolarization to 0.5 and 2 μM histamine (Fig. 7f), which might be due to direct LPI effect on ion channels other then BK_{Ca}. Nevertheless, these experiments clearly indicate that stimulatory effect of intracellular LPI on endothelial hyperpolarization to 0.5 and 2 μM histamine is partially attributed to stimulation of BK_{Ca} channels. These results confirm our findings in excised patches and indicate that LPI increases the

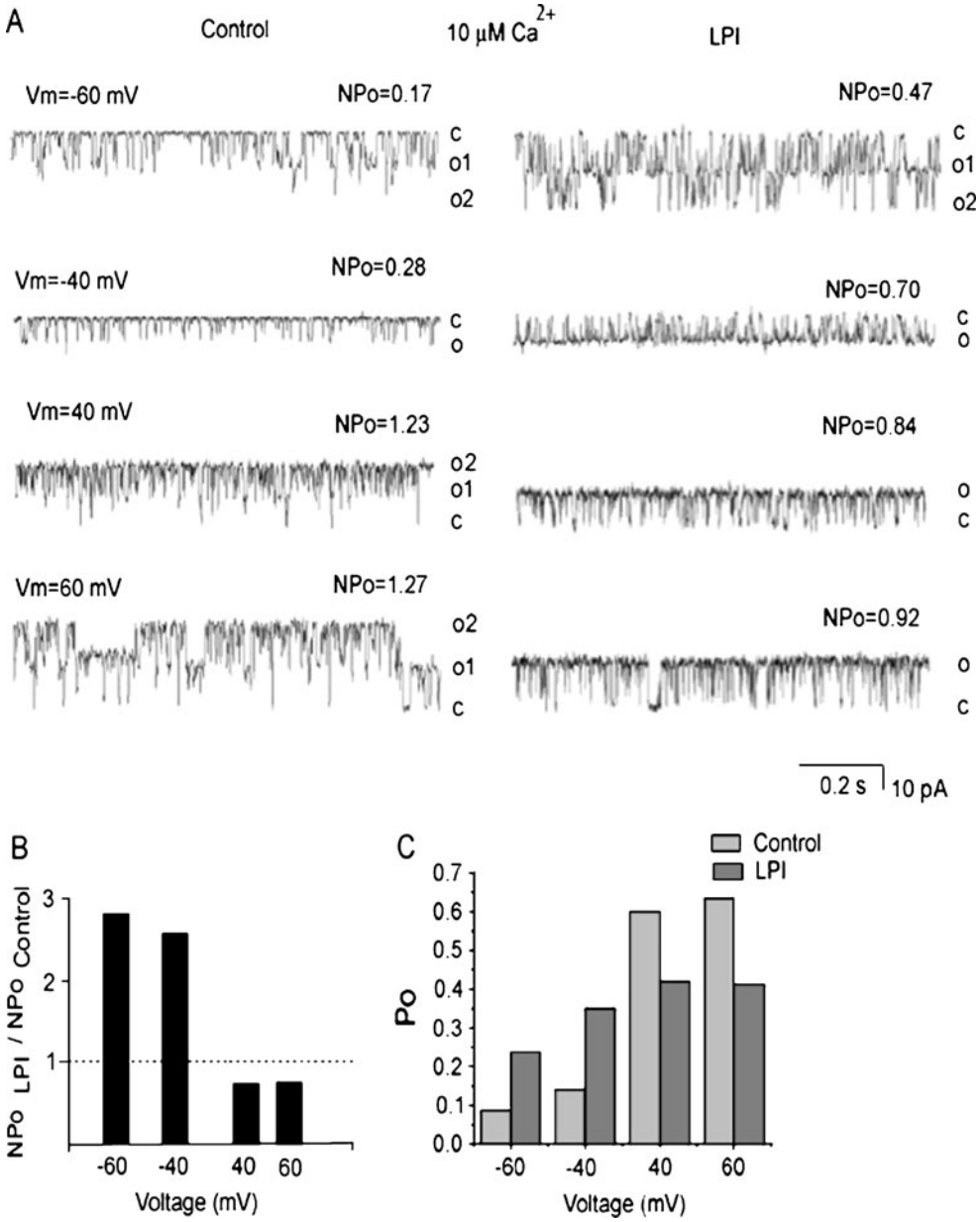

Fig. 6 The dual effect of LPI depends on the level of basal BK_{Ca} channel activity. **a** Representative single-channel recording in inside-out patch exposed to a single Ca^{2+} concentration of 10 μM at different voltages. LPI (3 μM) increases NPo at negative voltages (Vm=−40 and Vm=−60 mV) and decreases NPo at positive voltages (Vm=+40 and Vm=+60 mV), where NPo is high (*c*, *closed*, *o*, *open state*). **b** Graphical representation of the dual effect of LPI at different voltages in the same patch. **c** Graphical representation showing Po values in the absence (control) and presence of 3 μM LPI at different voltages in the same patch

sensitivity of endothelial cells to submicromolar concentrations of histamine by facilitating the Ca^{2+}-induced activation of BK_{Ca} channels.

To test the effect of LPI under maximal activation of endothelial cells, the cells were stimulated with 100 μM histamine in the absence or presence of 1 μM LPI in the pipette. Notably, 1 μM LPI in the pipette slightly reduced the endothelial cell peak hyperpolarization in response to supramaximal histamine concentration (i.e., 100 μM) from 34.9±1.5 mV (n=24) to 31.4±1.4 mV (n=40) (p=0.055) (Fig. 8a, b, d). To estimate the effect of intracellular LPI on the sustained component of endothelial hyperpolarization, comparison of the membrane potential recovery after the peak has been performed as described previously [3]. In the presence of intracellular LPI (1 μM), the membrane potential declined faster and hyperpolarization after 300 s was 71.7±4.7% of the peak level of −63.9±1.2 mV (n=11). In contrast, in the absence of LPI in the patch pipette, the hyperpolarization to 100 μM histamine after 300 s was 90.1±5.6% (Fig. 8e) of the peak level of −71±2.6 (n=5). In the combined presence of external iberiotoxin (100 nM) and internal LPI (1 μM), the sustained component of endothelial hyperpolarization was further decreased as evidenced from further reduction of hyperpolarization after 300 s to 58.2±8.7% (n=5) (Fig. 8c, e) from the peak level of −60.1±3.6 mV. These data support our findings presented in isolated patches and indicate that LPI indeed exhibits inhibitory properties on BK_{Ca} channel activity

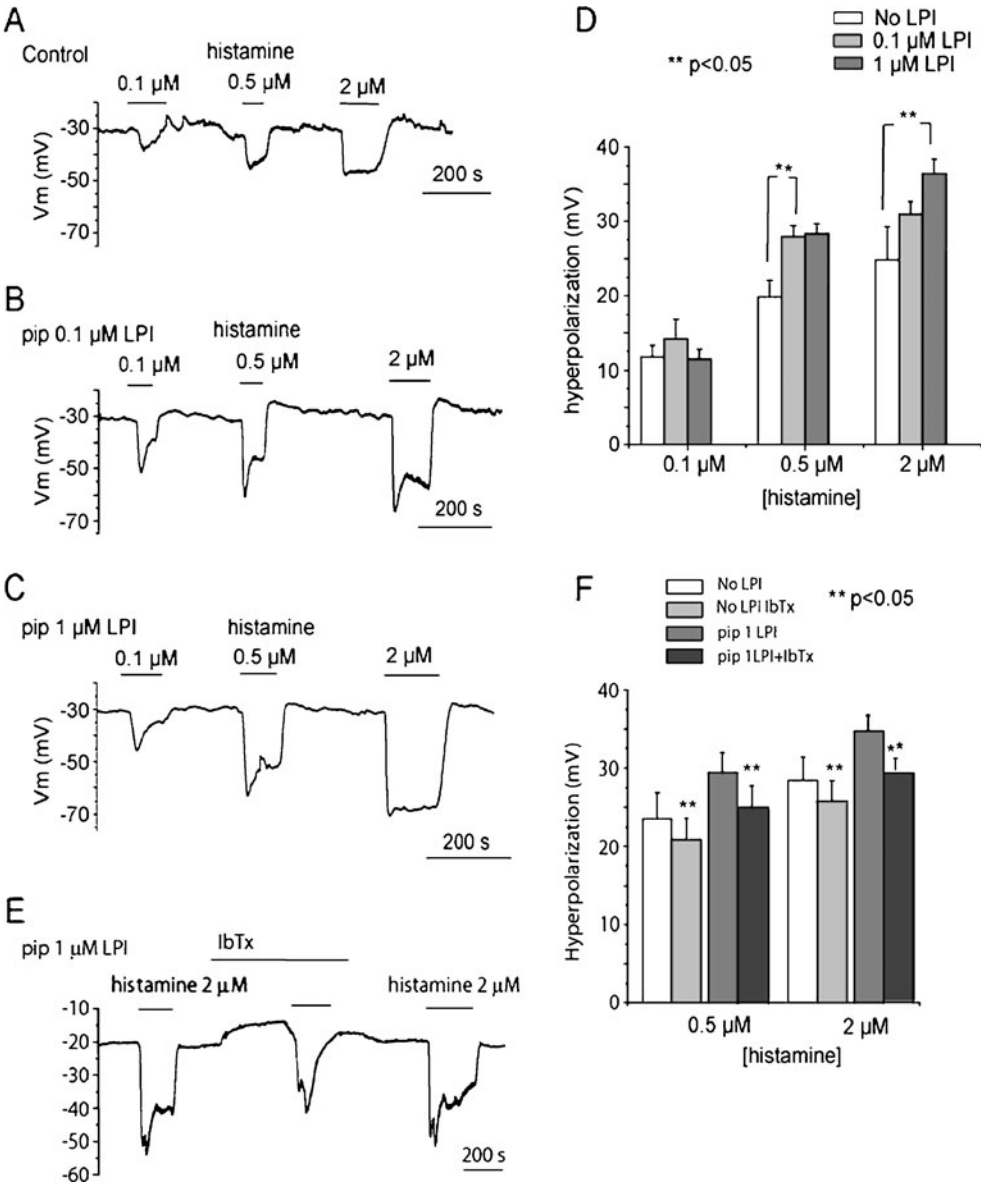

Fig. 7 Intracellular LPI potentiates endothelial cell hyperpolarization to low histamine concentrations through BK_{Ca} channels. **a**, **b**, **c** Representative endothelial cell hyperpolarization to bath application of 0.1, 0.5, and 2 μM histamine under control conditions (no LPI in patch pipette; **a**), or in the presence of 0.1 (**b**) and 1 μM LPI (**c**) in patch pipette. **d** Statistical representation of the hyperpolarizing effect of various moderate histamine concentrations (0.1, 0.5, and 2 μM) in the absence or presence of 0.1 or 1 μM LPI into patch pipette [No LPI, 0.1 (n=19), 0.5 (n=16), and 2 μM histamine (n=7); 0.1 μM LPI, 0.1 (n=10), 0.5 (n=10), and 2 μM histamine (n=7); 1 μM LPI, 0.1 (n=6), 0.5 (n=7), and 2 μM histamine (n=5)]. *p< 0.05 vs. the absence of LPI in the pipette. **e** Representative membrane potential recording showing inhibitory effect of iberiotoxin (100 nM) on potentiated by intrapipette LPI (1 μM) endothelial cell hyperpolarization to 2 μM histamine. **f** Statistical representation of the effect of iberiotoxin (100 nM) on endothelial cell peak hyperpolarization to 0.5 and 2 μM histamine with and without 1 μM LPI in the pipette; (histamine 0.5 μM−no LPI in pipette±iberiotoxin, n=8; 1 μM LPI in pipette±iberiotoxin, n=7; histamine 2 μM−no LPI in pipette±iberiotoxin, n=8, 1 μM LPI in pipette±iberiotoxin, n=11)

under conditions of excessive endothelial cell stimulation, thus, approving the concept of the dual modulator function of LPI in the vascular wall.

Discussion

Like in many other cell types, in endothelial cells, LPI triggers an increase of the intracellular Ca^{2+} concentration by either intracellular Ca^{2+} mobilization and/or the activation of Ca^{2+} influx that subsequently yields activation of BK_{Ca} channels [1, 21, 37, 38]. Very recently, these effects could be attributed to the binding of LPI to the orphan receptor GPR55 endogenously expressed in endothelial cells [42] and in HEK293 cells artificially expressing GPR55 [22–24]. Moreover, stimulation of endothelial cells with LPI was shown to be accompanied by an activation of BK_{Ca} channels that was thought to be a consequence of the LPI-elicited Ca^{2+} signals [4]. In addition, LPI was shown to affect directly the activity of ion channels, including members of the transient receptor potential channel family [14, 41], endothelial non-selective cation channels [4], and the two-pore domain mechano-gated TREK-1 and TRAAK K^+ channels [31]. However, so far, no evidence has been provided for a direct G protein-coupled receptor-independent modulation of BK_{Ca} channels by LPI.

The data presented herein demonstrate that LPI, in physiological relevant concentrations [10, 44], dually modifies endothelial BK_{Ca} channel activity in isolated inside-out patches as well as in current clamp experiments. Although the presence of Ca^{2+} was a prerequisite for the modulator effect of LPI on BK_{Ca} channels, the

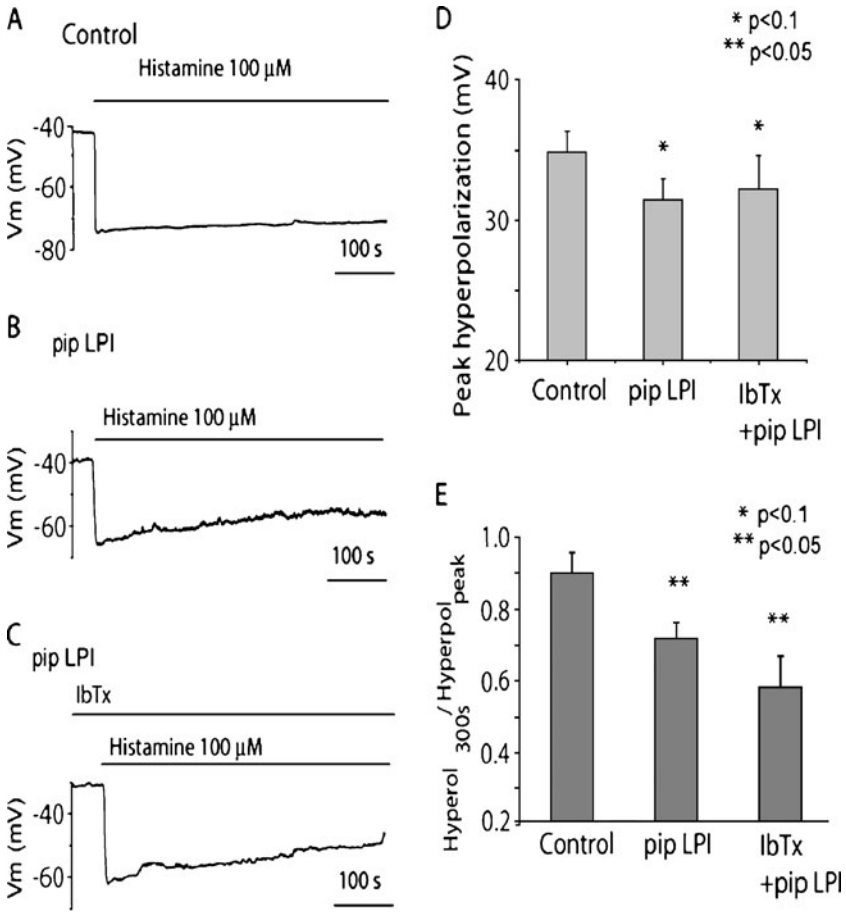

Fig. 8 Intracellular LPI counteracts endothelial cell hyperpolarization to supramaximal histamine concentrations. **a–c** Representative endothelial cell hyperpolarization to bath application of 100 µM histamine under control condition (**a**) and under condition of cell dialysis with 1 µM LPI via patch pipette in the absence (**b**) and presence (**c**) of external iberiotoxin (IbTx, 100 nM). **d** Statistical representation of the effect of cell dialysis with 1 µM LPI in the absence and presence of external 100 nM iberiotoxin (IbTx) on peak endothelial cell hyperpolarization to supramaximal histamine concentration (i.e., 100 µM). *$p<0.1$ vs. in the absence of LPI in the pipette and IbTx in the bath (control, $n=24$; LPI, $n=40$; IbTx+LPI, $n=9$). **e** Statistical representation of the effect of cell dialysis with 1 µM LPI in the absence and presence of external 100 nM iberiotoxin (IbTx) on sustained endothelial cell hyperpolarization to supramaximal histamine concentration (i.e., 100 µM). Results are expressed as the ratio of mean membrane potential values at 300th second after the peak and at the peak of hyperpolarization. **$p<0.05$ vs. in the absence of LPI in the pipette and IbTx in the bath (control, $n=24$; LPI, $n=40$; IbTx+LPI, $n=9$)

mode of the LPI-induced modulation of BK_{Ca} channel activity did not essentially depend on the actual Ca^{2+} concentration or voltage but strictly depended on the basal activity of the channel yielding either pronounced augmentation at low basal activity or attenuation if the basal BK_{Ca} channel activity was high. A change from LPI-mediated channel activation (increase in NPo) to channel inhibition occurs at a Po value of approximately 0.3. Because endothelial BK_{Ca} channels lack the regulatory β-subunit [34], the experiments using excised membrane patches suggest that LPI exhibits its effects by a direct interaction with the pore-forming component of these ion channels. While our findings in excised patches excludes the involvement of second messengers in the LPI-mediated modulation of BK_{Ca} channels, an immediate alteration of the proteolipid environment by LPI that might account for alterations of BK_{Ca} channel activity cannot be excluded. However, all effects of LPI on BK_{Ca} channels we have observed were sustained, concentration-dependent, and reversible. Importantly, LPI was used in low micromolar concentrations, thus, well below the LPI critical micellar concentration of ~75 µM [13], ensuring that the LPI actions on BK_{Ca} channels are caused by LPI monomers and are not due to nonspecific effects of LPI as a detergent. Furthermore, effective LPI concentrations are within the range found under normal (<0.1 µM) and pathological condition (~15 µM) in human tissue fluids [10, 44] and plasma [39].

The observation that both activation and inhibition of BK_{Ca} channel activity by LPI occurred in the same concentration range indicates that LPI has similar potency for both phenomena, though it remains unclear whether or not both effects correspond to distinct or identical interaction sites of LPI on the BK_{Ca} channel pore protein.

The dual effects of LPI on endothelial BK_{Ca} channels were observed both in multiple channel patches and in patches containing only one active channel, indicating that alterations in the channel activity may occur due to changes in the channel's Po rather then changes in the number of active channels per patch. Although the latter possibility cannot be entirely excluded, our observation that the level of potentiation of BK_{Ca} channel activity was quantitatively similar (p=0.34) in patches containing one and several active channels favors for the possibility that modulation of BK_{Ca} channel activity occurs without changes in the number of active channels per patch. Moreover, LPI did not affect single-channel current amplitude or its conductance, suggesting that LPI does not alter BK_{Ca} channel function by changing the entire conformation of the channel protein. Analysis of single-channel kinetics revealed that LPI acts primarily via alterations of the mean closed time, while the mean open time was only moderately affected, thus indicating that LPI modulates the activity of BK_{Ca} channels mainly via a destabilization of the closed states.

The current study describes LPI as an effective stimulator as well as inhibitor of endothelial BK_{Ca} channels. Considering that the action of endothelium-dependent vasodilators is mostly underpinned by a rise in the cytosolic Ca^{2+} concentration and membrane potential fluctuations, the dual modulation of endothelial BK_{Ca} channels by LPI might be of considerable physiological importance and may reflect a new mechanism of vascular function control by lysophospholipids.

This assumption is directly supported in experiments using the whole-cell recordings approach. In moderately stimulated cells that developed weak to modest hyperpolarization due to the increased cytosolic Ca^{2+}, the stimulatory effect of LPI on BK_{Ca} channels remained sustained and increased the peak hyperpolarization to histamine. Remarkably, not all of the stimulatory effect of internal LPI is inhibited by iberiotoxin, indicating that ion channel other then BK_{Ca} may partially underpin the stimulatory effect of LPI on endothelial hyperpolarization. Such stimulatory effect of endothelial K_{Ca} channels has been already reported by cAMP [18] and yielded elevated Ca^{2+} signaling and production of nitric oxide [19]. In contrast, under condition of excessive cell stimulation that leads to a strong Ca^{2+} loading, LPI was found to exhibit an inhibitory effect on endothelial hyperpolarization and, thus, counteracted the excessive cell stimulation. Because endothelial LPI production is controlled by cytosolic Ca^{2+} [2, 27], these data indicate that cytosolic LPI acts as dual modulator of endothelial hyperpolarizing response. Notably, in isolated rat aorta, sustained endothelial hyperpolarization to acetylcholine is partially mediated by stimulation of Na^+–K^+ ATPase [3], and LPI was recently shown to inhibit effectively Na^+–K^+ ATPase [4], raising the possibility that modulatory effect of internal LPI on endothelial electrical responses may be mediated via multiple mechanisms including ion channels and transporters.

Lipids are known to modify BK_{Ca} channel activity. In particular, negatively charged lipids and fatty acids were shown to stimulate the BK_{Ca} channel, while positively charged lipids with a sufficiently hydrophobic acyl chain suppress the channel activity in inside-out patches [36]. Controversial data regarding the effect of sphingosine on the BK_{Ca} channel have been reported with both stimulatory [26] and inhibitory [39] effects. These conflicting findings might point to a similar dual effect of sphingosine on BK_{Ca} channel activity than that of LPI reported herein. These findings may significantly extend our understanding of the effects of LPI on plasma ion channels and reveal a new feature of this compound of which the physiological contribution still remains elusive despite the release/existence of LPI in the vasculature/blood is approved. Whether such a dual modification of the activity of the BK_{Ca} channel is unique for LPI or exhibits a common feature for other LPLs awaits further investigations.

Strikingly, several substances including ethanol [29] and the xenoestrogen tamoxifen [35] were reported to dually affect BK_{Ca} channel activity. However, ethanol exerts its modulator effect on BK_{Ca} channels only at concentrations found in the circulation after excessive alcohol consumption (50–100 mM), and the effect of tamoxifen is only of clinical relevancy as this compound is therapeutically used as a competitive antagonist of the estrogen receptor. Nevertheless, among these agents that have a complex action on BK_{Ca} channels, LPI is unique as it serves as signaling molecule in the vasculature and can be found naturally in the vasculature/blood under physiological and pathophysiological conditions [10, 44].

Recently, LPI was shown to activate non-selective cation channels both when applied extracellularly or in excised inside-out membrane patches [4, 41]. Therefore, it appears reasonable that LPI may affect ion channels from the outer as well as the inner side of the plasma membrane. However, charged lipids are generally thought to act on the external side of the membrane affecting BK_{Ca} channels [7]. Therefore, BK_{Ca} channels may probably directly sense LPI from both intracellular and extracellular sides of the membrane. It is known that LPI levels increase almost threefold within seconds upon stimulation of endothelial cells with bradykinin [28]. Such an increase of the cellular LPI content is not specific for bradykinin but is a consequence of elevated cytosolic free Ca^{2+} [2]. Considering the dual effects of LPI on BK_{Ca} channel activity and electrical responses reported in this study, we suggested that intracellularly generated LPI should dually regulate endothelial electrical responses to endothelium-dependent vasodilators. Hence, LPI may serve as potent inter- and

intracellular signaling molecule modulating BK_{Ca} channels in the vasculature.

In conclusion, we provide new information regarding the effects of LPI on endothelial K^+ channels. We demonstrate a direct dual action of LPI on endothelial BK_{Ca} channel gating that does not require other cytosolic factors but depend on a cytosolic Ca^{2+} elevation. These results describe a novel mechanism of the action of LPI and point to LPI as a potential second messenger in endothelial cells. LPI functions as a direct receptor-independent dual modulator of BK_{Ca} channels in endothelial cells that impacts electrical responses to agonists and, thus, may affect Ca^{2+} entry via store-operated and agonist-induced Ca^{2+} entry pathways [17]. This signaling of LPI might allow a fine tuning of Ca^{2+} sensitive processes within vascular cells and hence essentially contribute to the control of adequate blood flows in various organs and tissues.

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