Direct voltage control of endogenous lysophosphatidic acid G-protein-coupled receptors in *Xenopus* oocytes

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Lysophosphatidic acid (LPA) G-protein-coupled receptors (GPCRs) play important roles in a variety of physiological and pathophysiological processes, including cell proliferation, angiogenesis, central nervous system development and carcinogenesis. Whilst many ion channels and transporters are recognized to be controlled by a change in cell membrane potential, little is known about the voltage dependence of other proteins involved in cell signalling. Here, we show that the InsP$_3$-mediated Ca^{2+} response stimulated by the endogenous LPA GPCR in *Xenopus* oocytes is potentiated by membrane depolarization. Depolarization was able to repetitively stimulate transient [Ca^{2+}]$_i$ increases after the initial agonist-evoked response. In addition, the initial rate and amplitude of the LPA-dependent Ca^{2+} response were significantly modulated by the steady holding potential over the physiological range, such that the response to LPA was potentiated at depolarized potentials and inhibited at hyperpolarized potentials. Enhancement of LPA receptor-evoked Ca^{2+} mobilization by membrane depolarization was observed over a wide range of agonist concentrations. Importantly, the amplitude of the depolarization-evoked intracellular Ca^{2+} increase displayed an inverse relationship with agonist concentration such that the greatest effect of voltage was observed at near-threshold levels of agonist. Voltage-dependent Ca^{2+} release was not induced by direct elevation of InsP$_3$ or by activation of heterotrimeric G-proteins in the absence of agonist, indicating that the LPA GPCR itself represents the primary site of action of membrane voltage. This novel modulation of LPA signalling by membrane potential may have important consequences for control of Ca^{2+} signals both in excitable and non-excitable tissues.

Abbreviations 2-APB, 2-aminoethyl diphenylborinate; GPCR, G-protein-coupled receptor; LPA, lysophosphatidic acid.

Introduction

G-protein-coupled receptors (GPCRs) are the largest family of cell surface proteins and play key roles in the activation of virtually all cell types (Pierce *et al.* 2002). Although GPCRs are not normally considered to be sensitive to changes in the cell membrane potential, evidence is emerging to support this concept in both non-excitable and excitable tissues (Marty & Tan, 1989; Ganitkevich & Isenberg, 1993; Mahaut-Smith *et al.* 1999; Ben Chaim *et al.* 2003; Martinez-Pinna *et al.* 2005; Billups *et al.* 2006; Liu *et al.* 2009; reviewed in Mahaut-Smith *et al.* 2008). The most extensively studied example of this phenomenon is the bipolar voltage control of P2Y$_1$

receptor-evoked Ca^{2+} release in the rat megakaryocyte, a non-excitable cell type lacking voltage-operated Ca^{2+} channels or ryanodine receptors (Mahaut-Smith *et al.* 2008). Evidence suggests that voltage is able to modulate InsP$_3$-dependent Ca^{2+} release primarily by acting at the level of the P2Y receptor rather than directly regulating either associated G-proteins or downstream signals (Martinez-Pinna *et al.* 2005). This conclusion is also supported by observations of voltage-dependent charge movements, analogous to the gating currents of ion channels, in muscarinic (m2 and m1) GPCRs, expressed in *Xenopus* oocytes (Ben Chaim *et al.* 2006). Voltage control of GPCR signalling represents an important mechanism whereby physiological voltage waveforms

(Martinez-Pinna *et al.* 2004) can modulate cellular signals initiated by chemical stimuli. Given the ubiquitous role of GPCRs in cellular physiology, an important question is the extent to which other receptors within this family of surface proteins are also sensitive to changes in the transmembrane potential. In the present study we have addressed this question for GPCRs stimulated by the agonist lysophosphatidic acid (LPA), which play important roles in a variety of physiological processes such as embryogenesis, vascular development and neurogenesis and pathophysiological processes such inflammation, pain, neurodegenerative diseases and cancer (Moolenaar *et al.* 2004; Ishii *et al.* 2004; Noguchi *et al.* 2009). LPA receptors are expressed in major excitable tissues such as the heart and the brain where they will be exposed to rapid changes in membrane potential during synaptic inputs and action potential firing and thus where voltage dependence may significantly influence receptor function.

Although classically used for studying heterologously expressed proteins (Miledi *et al.* 1989), *Xenopus laevis* oocytes possess a plethora of native membrane proteins, including LPA GPCRs, that can be easily studied (Kusano *et al.* 1977). In particular, many properties of the LPA GPCRs and characteristics of their signalling pathways were first derived from studies using oocytes (Fernhout *et al.* 1992; Tigyi & Miledi, 1992). Furthermore, LPA receptors in *Xenopus* oocytes show significant homology ($\sim$89% identity) to mammalian LPA_1 receptors (Kimura *et al.* 2001). Therefore, we consider the *Xenopus* oocyte as a good model to study the voltage dependence of LPA signals in a native environment. Activation of LPA GPCRs triggers several intracellular signalling pathways, leading to a variety of biologically important cellular responses. Many of these actions of LPA are mediated through $InsP_3$-dependent Ca^{2+} release from the intracellular stores, which induces an $[Ca^{2+}]_i$ increase. We now report that, during stimulation of native oocyte GPCRs by a wide range of LPA concentrations, membrane depolarization directly enhances the activity of these receptors, resulting in increased receptor-evoked $[Ca^{2+}]_i$, due to $InsP_3$-dependent Ca^{2+} release from intracellular stores.

Methods

Oocyte preparation and microinjection

Animal handling was carried out in accordance with the guidelines for care and use of laboratory animals adopted by the EU and with the standards of *The Journal of Physiology* (Drummond, 2009). Twenty adult female *Xenopus laevis* (purchased from Biological Blades, Edenbridge, Kent, UK) frogs were fully anaesthetized by immersion in cold 0.17% MS-222 for 15 min and

a piece of ovary was aseptically removed. Fully grown immature oocytes were isolated from the ovary and their surrounding layers removed manually, as previously described (Ivorra & Morales, 1997). Cells were kept at 15–16°C in a modified Barth's solution (mM): 88 NaCl, 1 KCl, 2.40 $NaHCO_3$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 10 Hepes, pH 7.4, supplemented with penicillin (100 units ml^{-1}) and streptomycin (0.1 mg ml^{-1}) until used for recordings. Oocytes were intracellularly microinjected with 50 nl of the fluorescent Ca^{2+} indicator K_5-Fluo5 (1 mM, final oocyte concentration 50 μM) at least 1 h before the recording, using a nanolitre injector (Nanoliter 2000, WPI, Stevenage, UK). $InsP_3$ was injected into the oocyte from a micropipette containing 1 mM $InsP_3$ using a pneumatic pressure ejection system (Pico-spritzer, General Valve, Fairfield, NJ, USA) with pulses of 35 p.s.i. and 30 ms duration. The volume of $InsP_3$ injected was $\sim$500, estimated from the diameter of the droplet expelled with the pipette tip in air, and would result in an intracellular concentration of $\sim$500 nM, assuming even distribution throughout the oocyte.

Solutions

Normal frog Ringer solution contained (mM): 115 NaCl, 2 KCl, 1.8 $CaCl_2$, 5 Hepes, pH 7.0. A few minutes after the beginning of the experiment the oocytes were usually superfused with Ca^{2+}-free external solution (mM): 115 NaCl, 2 KCl, 1.8 $MgCl_2$, 5 Hepes, 0.5 EGTA, pH 7.0 (see Results). Drugs were diluted in the appropriate external solution and applied by superfusing the oocyte at a flow rate of 7–10 ml min^{-1}. Cells were exposed to AlF_4^- by the addition of 10 mM NaF and 200 μM $AlCl_3$ to the external saline. K_5-Fluo5 was purchased from Invitrogen (Eugene, OR, USA), $InsP_3$, stocked in aqueous solution including 50 μM EDTA and 5 mM Hepes at pH 7.0, from Calbiochem (San Diego, CA, USA) and the rest of reagents from either Sigma (St Louis, MO, USA) or Sharlau Chemie SA (Barcelona, Spain). Oocytes were kept in the dark during incubation with U-73122 or U-73343 for the periods shown in Fig. 4*B*.

Electrophysiological recordings

Membrane current recordings were performed at 21–25°C, 1–10 h after K_5-Fluo5 injection, using a high-compliance two-microelectrode voltage-clamp system (TurboTEC-10CD npi, Tamm, Germany). Intracellular electrodes (1–3 MΩ) were filled with 3 M KCl and 3 M potassium acetate for voltage recording and current injection, respectively. Oocytes were placed in a 150 μl recording chamber that was continuously superfused with the appropriate external solution. The membrane potential was held at -80 mV and voltage

steps to -10 mV were applied for a duration of 60 s, unless otherwise stated. Membrane currents were low-pass filtered at 30–200 Hz and recorded on a PC, after sampling (Digidata 1200, Molecular Devices, Sunnyvale, CA, USA) at fivefold the filter frequency, using the WCP v.3.2.8 package developed by J. Dempster (Strathclyde Electrophysiology Software, University of Strathclyde, UK).

Fluorescence recordings

Fluorescence measurements were conducted using an epifluorescence system coupled to a stereomicroscope MZ12 (Leica Microsystems AG, Wetzlar, Germany). Light from a mercury arc lamp (LEJ, Jena, Germany) passed through a 470/40 nm bandpass filter to excite the fluorophore. Emitted light (>515 nm) was selected using a dichroic mirror and monitored without further filtering by a photomultiplier tube (Hamamatsu Photonics, Tokyo, Japan). Fluorescence signals were acquired using WCP v.3.2.8 software, sampled at 30–100 Hz and exported for analysis with Origin (OriginLab Corp., Northampton, MA, USA). For presentation, traces were filtered using 5–20 point averaging. LPA or voltage steps were considered to induce a response when the fluorescence changed by $\geq 3\%$ from the prestimulus level. Fluo-5 fluorescence signals are expressed as ratios (f/f_0), where f_0 is the basal fluorescence level prior to the stimulus (agonist or depolarization). The amplitude of responses to different stimuli was measured as the peak change in the f/f_0 response ($\Delta f/f_0$). Experiments used oocytes with a similar baseline fluorescence level (f_0), indicating a constant intracellular concentration of K_5-Fluo5. To minimize variations due to hemispheric asymmetry in Ca^{2+} responses mediated through receptors coupled to $InsP_3$-dependent Ca^{2+} release (Miledi & Parker, 1989) and also to avoid light absorption by pigment in the animal hemisphere, oocytes were positioned such that the vegetal (translucent) hemisphere faced the light source.

Data analysis

Averaged data are expressed as the mean $\pm$ S.E.M. and statistical difference was tested using Student's unpaired t test. A level of $P < 0.05$ was considered significant.

Results

Voltage-dependent $[Ca^{2+}]_i$ modulation during LPA receptor stimulation

In oocytes perfused with normal Ringer solution, depolarization from a holding potential of -80 mV to -10 mV had no effect on $[Ca^{2+}]_i$ ($n = 20$; Fig. 1A–C

and F), consistent with a lack of voltage-dependent Ca^{2+} influx over this range of potentials. Thus, although voltage-gated Ca^{2+} channels are occasionally observed in *Xenopus* oocytes (Miledi, 1982), their contribution to the Ca^{2+} responses in the oocytes used in the present study was negligible. At a constant holding potential of -80 mV, application of 10 nM LPA evoked a transient increase in $[Ca^{2+}]_i$ (peak $= 0.66 \pm 0.08$ $\Delta f/f_0$, $n = 20$; Fig. 1A), that slowly decayed to basal levels in the continued presence of agonist. As can be seen by comparing Fig. 1A–C, the magnitude and rate of decay of the Ca^{2+} response to 10 nM LPA displayed significant intercellular heterogeneity, but in all cells consisted of a single peak, which then decayed at a variable rate. In contrast to the unstimulated condition, depolarization to -10 mV during the LPA response evoked a marked, transient $[Ca^{2+}]_i$ increase in 75% of the oocytes tested (16 out of 20; peak 0.30 ± 0.05 $\Delta f/f_0$, Fig. 1B and F) and a small $[Ca^{2+}]_i$ decrease in the remaining 25% (4 out of 20; peak -0.09 ± 0.02 $\Delta f/f_0$, Fig. 1C). A correlation existed between the magnitude of the response to LPA and the polarity and amplitude of the effect of depolarization ($R = -0.63$; $P < 0.01$, supplementary Fig. 1). Depolarization during small or average amplitude responses to 10 nM LPA generated an increased $[Ca^{2+}]_i$ (Fig. 1B), but produced a fall in $[Ca^{2+}]_i$ during larger agonist-evoked responses (Fig. 1C). Thus, the heterogeneity in the response to a voltage step can be explained by an increasing contribution of agonist-evoked Ca^{2+} influx as the LPA-mediated response becomes larger, since depolarization will reduce the driving force for Ca^{2+} entry. In the absence of external Ca^{2+}, the average response to 10 nM LPA was similar in magnitude to the control situation (peak 0.81 ± 0.09 $\Delta f/f_0$, $n = 20$, $P = 0.37$; Fig. 1D), as expected for a response that initially depends primarily on $InsP_3$-dependent Ca^{2+} mobilization. Depolarization was still able to generate an increase of $[Ca^{2+}]_i$, and importantly this was observed in 100% of oocytes (20/20) (peak 0.17 ± 0.02 $\Delta f/f_0$, $n = 20$; Fig. 1D and F). The average response to depolarization was slightly reduced compared to normal Ca^{2+}-containing saline ($P < 0.05$; Fig. 1F), which may result from a reduced content of the Ca^{2+} stores after the initial LPA response. Nevertheless, in cells exposed to Ca^{2+}-free medium, sufficient Ca^{2+} store content was available to observe multiple depolarization-dependent Ca^{2+} increases in the presence of LPA (Fig. 1D). The experiment in Fig. 1D also shows that the induction of these transient depolarization-evoked Ca^{2+} increases by LPA was readily reversed by removal of agonist. To assess whether the depolarization-dependent Ca^{2+} increase observed during stimulation by LPA was the result of a voltage modulation of the electrogenic Na^+–Ca^{2+} exchanger, this protein was blocked by 5 mM extracellular Ni^{2+} (Schlief & Heinemann, 1995). Ni^{2+} did not block the depolarization-evoked $[Ca^{2+}]_i$ increase ($n = 6$, Fig. 1F) induced by LPA receptor
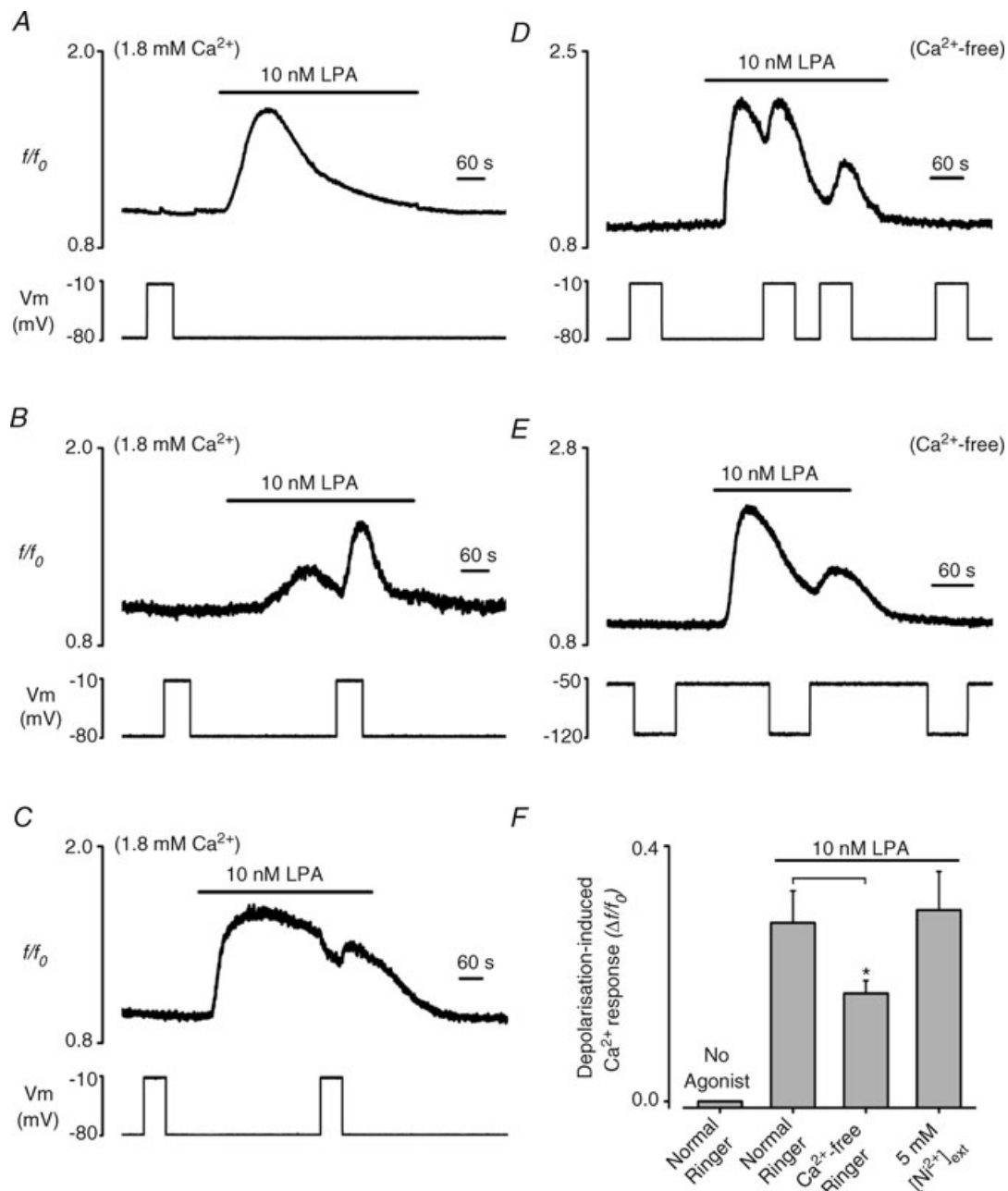
Figure 1. Effect of membrane voltage on LPA-evoked Ca^{2+} responses in *Xenopus* oocytes

A–E, intracellular Ca^{2+} recordings from oocytes under two-electrode voltage clamp in normal (Ca^{2+}-containing) Ringer solution (*A–C*) or Ca^{2+}-free Ringer solution (*D* and *E*). Upper panels show the Fluo5, f/f_0 ratio and lower panels the membrane voltage. *A*, typical recording showing the lack of effect of a 70 mV depolarizing voltage step (-80 to -10 mV, lower panel) on [Ca^{2+}]$_i$ (top panel) in the absence of agonist and the typical response to 10 nM LPA held at -80 mV. *B* and *C*, typical recordings of the [Ca^{2+}]$_i$ increase (*B*, 75% of cells) and [Ca^{2+}]$_i$ decrease (*C*, 25% of cells) evoked by depolarization during exposure to LPA. *D*, recording showing the repetitive and reversible nature of the depolarization-evoked Ca^{2+} increase observed in 100% of oocytes in Ca^{2+}-free medium. *E*, effect of membrane hyperpolarization (-50 to -120 mV) on the response to 10 nM LPA. *F*, bar graph showing the average depolarization-induced Ca^{2+} response ($\Delta f/f_0$) in the absence (no agonist) or presence of 10 nM LPA in the presence and absence of external Ca^{2+} and in the presence of 5 mM Ni^{2+} in Ca^{2+}-free Ringer solution. The depolarization-evoked response in Ca^{2+}-free Ringer solution was significantly smaller than that observed in normal Ringer solution (asterisk, $P < 0.05$).

stimulation in a Ca^{2+}-free medium, indicating that altered Ca^{2+} efflux via the Na^+–Ca^{2+} exchanger is not required.

Together, these data indicate a major role for Ca^{2+} release from intracellular stores in the depolarization-evoked increase of $[Ca^{2+}]_i$ during stimulation of LPA receptors. Furthermore, it suggests that the depolarization-evoked $[Ca^{2+}]_i$ decrease observed in the presence of external Ca^{2+} in a proportion of oocytes was due to a reduction of the driving force for Ca^{2+} entry. The final change in $[Ca^{2+}]_i$ in response to a depolarizing voltage step will vary in individual cells as a result of the balance between the depolarization-induced Ca^{2+} release and the reduction of the driving force for Ca^{2+} entry. For this reason, all subsequent experiments, which are aimed at investigating the properties and mechanism of the voltage-dependent Ca^{2+} release phenomenon, were performed in Ca^{2+}-free external solutions.

Previous studies showing voltage control of Ca^{2+} mobilization via native P2Y and $G\alpha_q$-coupled muscarinic receptors have demonstrated that the effect is bipolar in nature (for review see Mahaut-Smith *et al.* 2008), such that membrane hyperpolarization causes a transient reduction in the agonist-evoked response. During stimulation of LPA receptors in *Xenopus* oocytes, the effect of hyperpolarization was less obvious, probably due to the transient nature of the agonist-evoked $[Ca^{2+}]_i$ increase

and the relatively slow speed of the voltage-dependent response compared to previous studies (see Fig. 1*E*, where a 70 mV hyperpolarizing pulse was applied during the LPA-mediated Ca^{2+} increase). However, in contrast to P2Y receptor-evoked responses in the megakaryocyte (Martinez-Pinna *et al.* 2005), the level of the steady holding potential had a marked effect on the initial Ca^{2+} response stimulated by LPA in oocytes. Overall, a shift of holding potential to a more depolarized level accelerated and amplified the agonist-evoked response. For example, the latency of the onset of a 10 nM LPA-evoked response was shorter at −20 mV compared to at −80 mV (14 ± 1 s *vs.* 39 ± 4 s, $n = 9$, $P < 0.05$; Fig. 2*A–C*). Furthermore, the time to peak of the response to 10 nM LPA was less at −20 mV compared to at −80 mV (40 ± 1 s *vs.* 62 ± 6 s, $n = 9$, $P < 0.05$; Fig. 2*A*, *B* and *D*). Finally, 10 nM LPA stimulated a larger peak Ca^{2+} increase at a holding voltage of −20 mV compared to at −80 mV (1.78 ± 0.1 $\Delta f/f_0$ *vs.* 1.31 ± 0.1 $\Delta f/f_0$, $n = 9$, $P < 0.05$; Fig. 2*A*, *B* and *E*), with an intermediate amplitude increase being observed at −50 mV (1.56 ± 0.16 $\Delta f/f_0$, $n = 8$, Fig. 2*E*). These results demonstrate that the voltage control of LPA-induced Ca^{2+} responses is substantially modulated in time course and amplitude over a physiologically relevant range of steady membrane potential, the response being enhanced and accelerated by a depolarizing shift in voltage.
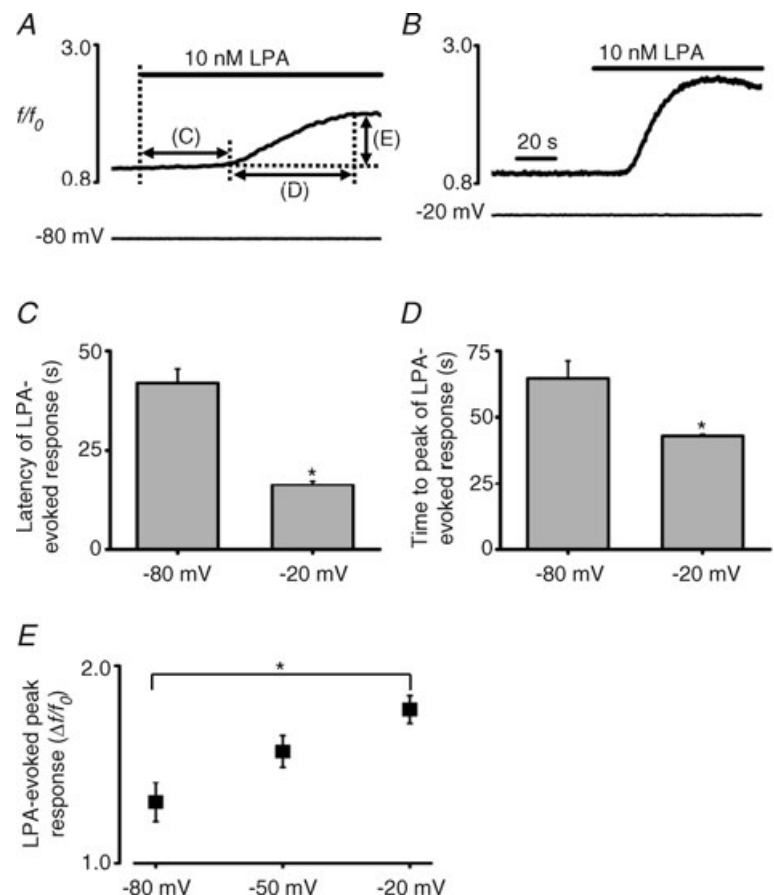

Figure 2. Effect of the holding potential on LPA-evoked Ca^{2+} responses
A and *B*, representative traces showing the influence of the holding potential on LPA-evoked Ca^{2+} release at −80 and −20 mV, respectively, in a Ca^{2+}-free external solution. Time scale in *A* is the same as in *B*. The lines with arrows in *A* indicate the parameters quantified in panels *C*, *D* and *E*. The latency (*C*), the time to peak (*D*) and the amplitude (*E*) of LPA-evoked Ca^{2+} response were measured in 8–9 oocytes at the constant holding potential shown (−80, −50 and −20 mV). The values for the three parameters were significantly different (asterisk, $P < 0.05$) between holding potentials of −80 and −20 mV.

Effect of agonist concentration on the voltage-dependence of LPA receptor-evoked Ca^{2+} release

The experiments above have explored the effect of membrane potential changes during application of a single concentration of LPA of 10 nM, which is close to the reported EC_{50} value for Ca^{2+}-dependent chloride current responses in *Xenopus* oocytes (Liliom *et al.* 1996) and close to the EC_{50} for Ca^{2+} signals in Jurkat T cells co-transfected with human LPA_1 or LPA_2 receptors and aequorin (An *et al.* 1998). The relationship between LPA concentration and LPA-dependent Ca^{2+} response in *Xenopus* oocytes was fitted with a sigmoid curve (Fig. 3D), with an estimated EC_{50} close to 100 nM. Depolarization-evoked Ca^{2+} increases were observed following exposure to a very wide range of LPA concentrations (Fig. 3B–D). Interestingly, the depolarization-dependent Ca^{2+} increase displayed an inverse relationship with LPA concentration, such that depolarization was more efficient at lower levels of receptor activation. The ratio of the $[Ca^{2+}]_i$ increase elicited by depolarization to that evoked by agonist was 0.74 ± 0.05, 0.58 ± 0.06, 0.36 ± 0.05, 0.14 ± 0.03 and 0.08 ± 0.02 fold at 1 nM, 3 nM, 5 nM, 10 nM and 50 nM LPA, respectively (Fig. 3D). At higher concentrations of agonist, 100 nM, 1 μM and 10 μM LPA the efficiency of depolarization to potentiate LPA response was lower than 0.05-fold (Fig. 3D). A further striking observation was that, although all cells (8/8) failed to respond to 0.1 nM LPA, the lowest concentration of LPA tested, three of these (37.5%) displayed a marked depolarization-evoked $[Ca^{2+}]_i$ increase (Fig. 3A). These results demonstrate that depolarization is most effective at modifying LPA-induced Ca^{2+} release at agonist concentrations in the nanomolar range, which, importantly, corresponds to the LPA levels found in plasma (Baker *et al.* 2001).

Requirement of functional InsP_3 receptors and phospholipase-C activation for the voltage control of Ca^{2+} release

Activation of LPA receptors in *Xenopus* oocytes is coupled to the phosphatidylinositol second messenger system (Tigyi & Miledi, 1992). To investigate whether functional InsP_3 receptors are required for the voltage-dependent Ca^{2+} release mechanism, experiments were undertaken with 2-aminoethyl diphenylborinate (2-APB), an inhibitor of InsP_3 receptors (Chorna-Ornan *et al.* 2001). Depolarization to -10 mV during the LPA-induced $[Ca^{2+}]_i$ transient (peak 0.60 ± 0.10 $\Delta f/f_0$, $n = 6$; Fig. 4A) evoked an increase in $[Ca^{2+}]_i$ in six oocytes (peak 0.13 ± 0.01 $\Delta f/f_0$, $n = 6$; Fig. 4A), but this response was abolished in the presence of 100 μM 2-APB (Fig. 4A), indicating the requirement of functional InsP_3 receptors for the voltage modulation of Ca^{2+} release.

Although 2-APB is widely used as an inhibitor of InsP_3 receptors, it may interfere with a variety of ion channels and pumps (Parekh & Putney, 2005). Hence, we further assessed the requirement of InsP_3 production in the voltage-dependent Ca^{2+} release during LPA receptor stimulation by incubation of oocytes with the aminosteroid U-73122 (1-[6-[((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione), an inhibitor of phospholipase-C activation (Noh *et al.* 1998). U-73122 (10 μM) decreased the response to depolarizing steps in the presence of LPA in a time-dependent manner (Fig. 4B). In these experiments, LPA was applied immediately prior to the depolarization; as expected, the response to LPA decreased over a similar time scale to the loss of response to depolarization (data not shown). In contrast,
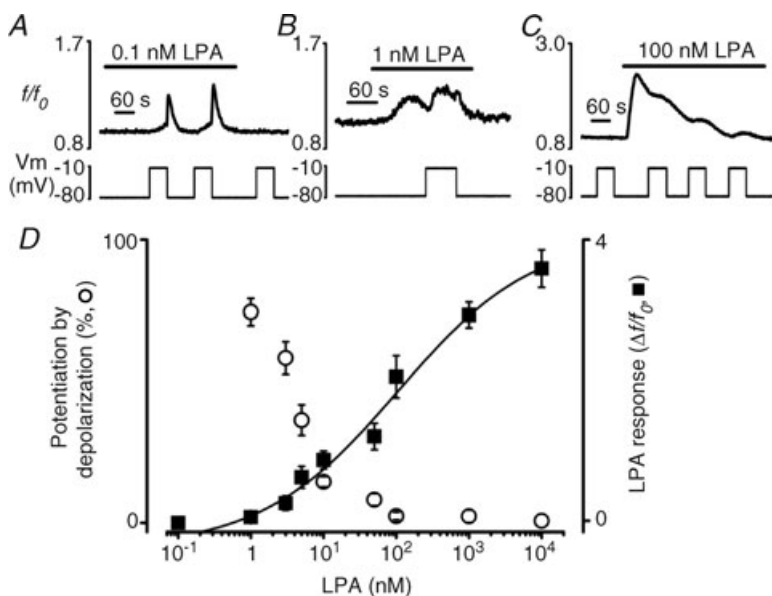

Figure 3. Potentiation of LPA receptor-evoked Ca^{2+} response by depolarization is greatest at low concentrations of LPA
A–C, effect of a depolarizing pulse (-80 to -10 mV) on $[Ca^{2+}]_i$ at a subthreshold (0.1 nM, A), near-threshold (1 nM, B) and suprathreshold (100 nM, C) concentration of LPA in Ca^{2+}-free external solution. At 0.1 nM LPA (A), all oocytes failed to respond to the agonist alone, whereas 3 out of 8 cells displayed a subsequent depolarization-evoked $[Ca^{2+}]_i$ increase. D, semilogarithmic concentration–response relationship for the depolarization-evoked $[Ca^{2+}]_i$ increase (open circles, measured as a percentage of the corresponding LPA-evoked response) and LPA-evoked peak response ($\Delta f/f_0$, filled squares), *versus* the LPA concentration. Potentiation by depolarization at 0.1 nM LPA is excluded due to lack of agonist response. LPA-evoked peak response data were best fitted with the logistic equation: $Y = \frac{A_1 - A_2}{1 + ([x/x_0])p} + A_2$, where x_0 is the middle value, p is the power and A_1 and A_2 are the initial and final Y values, respectively. Each point is the mean and S.E.M. of 4–13 cells.

incubation with the inactive analogue U-73343 (1-[6-[(((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-2,5-pyrrolidinedione; 10 μM) had no significant effect on the $[Ca^{2+}]_i$ increase elicited by either LPA (not shown) or by voltage steps in the presence of LPA (Fig. 4B). This indicates that activation of phospholipase-C, and thus $InsP_3$ production, plays an essential role in the voltage-dependent Ca^{2+} release during LPA receptor stimulation.

Lack of voltage-dependent Ca²⁺ release after direct elevation of InsP₃ or heterotrimeric G-protein activation

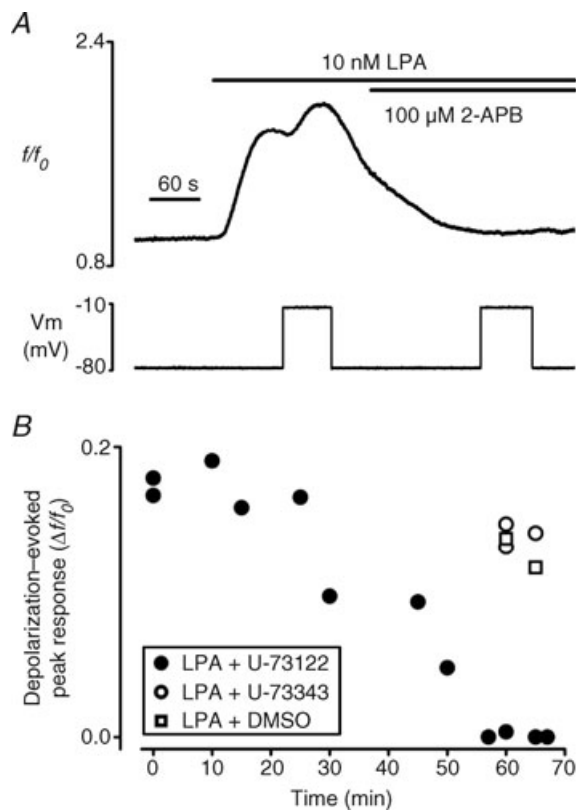The voltage dependence of Ca^{2+} release during activation of a GPCR can be explained by a direct sensitivity to membrane potential of the receptor itself or of any downstream molecules activated in the second messenger cascade (Ganitkevich & Isenberg, 1993; Mahaut-Smith et al. 1999; Ben Chaim et al. 2003; Martinez-Pinna et al. 2005; Ben Chaim et al. 2006). To assess whether activation of $InsP_3$ receptors alone can induce a voltage dependence of Ca^{2+} release in our cell model, for example by configurational coupling between $InsP_3$ receptors on the intracellular stores and ion channels on the plasma membrane (Kiselyov et al. 1998), we tested the effects of directly elevating the cytosolic $InsP_3$ level by pressure injection (see Methods). The pressure and duration applied to the intracellular micropipette were adjusted so that a single bolus of 1 mM $InsP_3$ generated a Ca^{2+} response (peak 0.80 ± 0.16 $\Delta f/f_0$, $n = 5$; Fig. 5A) of similar magnitude to that evoked by 10 nM LPA, a concentration at which depolarization-evoked Ca^{2+} increases are of a sizeable amplitude (see above). During this $InsP_3$-evoked response, depolarization was unable to stimulate a further Ca^{2+} increase (Fig. 5A), excluding the possibility that the voltage dependence during LPA
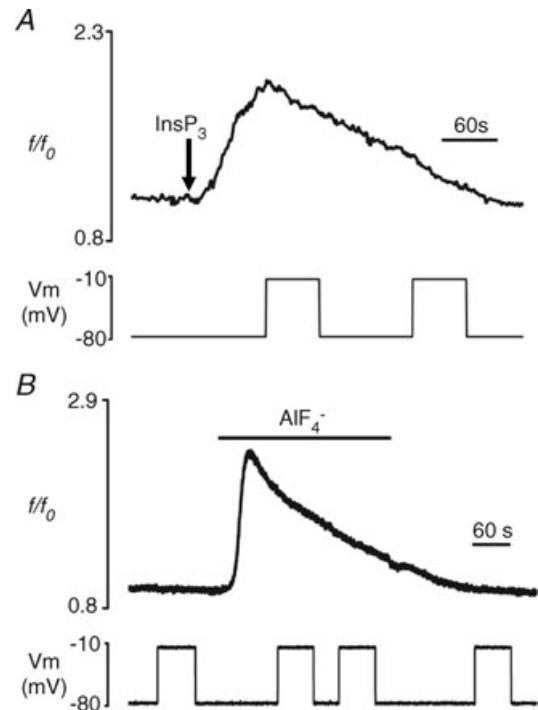

Figure 4. Functional InsP₃ receptors and phospholipase-C activation are required for the voltage control of Ca²⁺ release during LPA receptor stimulation
A, effect of depolarization (−80 to −10 mV) on $[Ca^{2+}]_i$ in the presence of 10 nM LPA alone (first voltage step) or following co-application of 100 μM 2-APB, an inhibitor of $InsP_3$ receptors (second voltage step). B, amplitude of the depolarization-evoked Ca^{2+} response (−80 to −10 mV, 60 s) in the presence of LPA after incubation with the phospholipase C inhibitor U-73122 (10 μM, filled circles), for different durations or following exposure to either its inactive analogue U-73343 (10 μM, open circles) or to DMSO at the same concentration used to dissolve these drugs (1/100 dilution, open triangles), for the duration required to observe the maximal effect with U-73122 (60–65 min). All experiments were performed in Ca^{2+}-free external solution. Each point represents a different cell.

Figure 5. Lack of depolarization-evoked Ca²⁺ response following direct elevation of InsP₃ or the heterotrimeric G-protein activation with aluminium tetrafluoride
A, lack of effect of depolarizing pulses (lower panel) on $[Ca^{2+}]_i$ (top panel) during the response to ~500 fmol of intracellularly injected $InsP_3$ (see Methods). Arrow indicates the point of $InsP_3$ injection. B, cells were exposed to the heterotrimeric G-protein activator AlF_4^- using a mixture of 10 mM NaF and 200 μM $AlCl_3$. Exposure to AlF_4^- reversibly induced a $[Ca^{2+}]_i$ increase (top panel) but depolarizing pulses (lower panel) had no effect on $[Ca^{2+}]_i$. All experiments were performed in Ca^{2+}-free external solution.

GPCR activation results from an event induced by direct activation of InsP_3 receptors or a subsequent step in the signalling cascade.

Extracellular application of aluminium tetrafluoride is employed as a tool to activate heterotrimeric G-proteins in the absence of agonist (Sternweis & Gilman, 1982; Martinez-Pinna *et al.* 2005). Superfusion of oocytes with AlF$_4^-$ resulted in an increase in [Ca^{2+}]$_i$ (peak 0.99 ± 0.08 $\Delta f/f_0$, $n = 8$; Fig. 5B), as a consequence of Gα_q-subunit activation and hence phospholipase-C stimulation and InsP_3 production (Moon *et al.* 1997). However, as observed following direct injection of InsP_3, depolarizing pulses were unable to stimulate a Ca^{2+} increase during AlF$_4^-$-induced responses (Fig. 5B). This indicates that the primary site of action of membrane voltage in the control of LPA GPCR-dependent Ca^{2+} mobilization is upstream of the heterotrimeric G-proteins.

Discussion

In this study we have demonstrated for the first time a sensitivity of LPA receptors to physiological changes in membrane potential. This extends previous work demonstrating an ability of membrane potential to directly modulate Ca^{2+} signalling via a number of GPCRs coupled to Gα_q-proteins and the phosphatidylinositol second messenger system. InsP_3-induced intracellular Ca^{2+} release mediates the action of many hormones, neurotransmitters and other messengers (Berridge, 1993; Clapham, 1995), including LPA, which plays a principal role in fundamental biological processes such as development, differentiation, angiogenesis, pain inflammation and carcinogenesis (Moolenaar *et al.* 2004; Ishii *et al.* 2004; Noguchi *et al.* 2009). Therefore, the fact that LPA signalling can be modulated by membrane potential may have important functional consequences. Of particular interest is the fact that LPA receptor expression is regulated throughout brain development (Fukushima *et al.* 2001) and that LPA induces cortical growth and folding (Kingsbury *et al.* 2003). Thus, the modulation of LPA signalling by voltage could be particularly relevant during neuronal growth and differentiation in the developing nervous system, where changes in the level of membrane potential are constantly occurring in the form of synaptic and action potentials. Furthermore, LPA is an inducer of cell proliferation, migration and survival and its plasma levels are increased in malignant secretions, indicating a role for LPA in the initiation and progression of cancer (Mills & Moolenaar, 2003). This, together with the increased expression of voltage-sensitive ion channels associated with a malignant potential (Fiske *et al.* 2006), makes the voltage dependence of LPA signals a factor to be considered in cancer pathophysiology. Voltage control of LPA receptors could also influence development, since

this agonist is known to control cortical actin assembly and cytoarchitecture in *Xenopus* embryos (Lloyd *et al.* 2005).

Two LPA receptor subtypes are expressed in *Xenopus* oocytes, XLPA$_1$-1 and XLPA$_1$-2, which are both highly homologous (∼89% identity) to mammalian LPA$_1$ receptors (Kimura *et al.* 2001). Indeed, the LPA$_1$ receptor shows a high degree of sequence conservation among many animals, including fish, amphibia (e.g. *Xenopus*), chicken and mammals (Chun *et al.* 2002). It is therefore possible that LPA receptors from different phyla show the voltage dependence. We have performed preliminary experiments in a human cell type (HEK-293 cells) which endogenously expresses LPA receptors to address this issue. In simultaneous whole-cell patch clamp and fura-2 fluorescence recordings, depolarization in the absence of agonist caused either no change, or a small reduction in Ca^{2+}. However, in the presence of 1 μM LPA, depolarizations were able to repeatedly stimulate an increase in [Ca^{2+}]$_i$ (supplementary Fig. 2 and unpublished results). This result indicates that the voltage dependence of Ca^{2+} signals during activation of LPA receptors is not restricted to lower vertebrates but is a widespread phenomenon.

Previous studies in a variety of cell types support the concept that voltage modulation is a common property of a number of GPCRs (Mahaut-Smith *et al.* 2008). Our results also suggest that the voltage sensor in the control of LPA GPCR-dependent Ca^{2+} release lies at the level of the receptor protein itself, since activation of G-proteins by aluminium tetrafluoride, in the absence of agonist, fails to induce voltage modulation of Ca^{2+} release. Moreover, InsP_3 receptors or events downstream of InsP_3-dependent Ca^{2+} release are not the voltage-sensing site, because direct intracellular injection of InsP_3 causes a [Ca^{2+}]$_i$ increase that cannot be modulated by voltage. Nevertheless, depolarization-induced Ca^{2+} release requires InsP_3 production and InsP_3-dependent Ca^{2+} release, as block of InsP_3 receptors with 2-APB (Chorna-Ornan *et al.* 2001) or inhibition of phospholipase-C activity abolished the response to LPA and the depolarization-induced Ca^{2+} release. These results indicate the essential role of the phosphatidylinositol second messenger system and that the modulation is upstream of InsP_3 production. A similar conclusion was derived in previous work on P2Y$_1$ and muscarinic receptors in megakaryocytes and smooth muscle, respectively, which is consistent with the hypothesis that depolarization stimulates InsP_3 production during activation of these GPCRs (Ganitkevich & Isenberg, 1993; Mahaut-Smith *et al.* 1999; Martinez-Pinna *et al.* 2005). Direct sensitivity of the GPCR to the membrane potential has been suggested by a series of observations on P2Y$_1$ receptors and also by observations of voltage-dependent charge movements, analogous to gating currents of voltage-gated channels, in two prototypical GPCRs, the m2 and m1 muscarinic

receptors, expressed in *Xenopus* oocytes (Ben Chaim *et al.* 2006). In the proposed mechanism, the intracellular loop that couples m2 and m1 receptors to their G-protein has a crucial function in voltage sensing, thereby influencing the affinity of the receptor for the agonist. On the other hand, any putative role of the electrogenic Na^+–Ca^{2+} exchanger in the voltage modulation of $[Ca^{2+}]_i$ during LPA application was ruled out in our experiments by superfusing the cell with a Ca^{2+}-free Ringer solution in the presence of Ni^{2+}, a blocker of the electrogenic Na^+–Ca^{2+} exchanger. To date, all GPCRs shown to exhibit voltage dependence fall into class A, which constitutes ~90% of all members of this superfamily of receptors (Pierce *et al.* 2002), including purinergic $P2Y_1$, thromboxane A_2, serotoninergic 5-HT_{2A}, glutamatergic mGlu3 and mGlu1, muscarinic m2, m1 and m3, dopaminergic D_2 (Ganitkevich & Isenberg, 1993; Ben Chaim *et al.* 2003; Martinez-Pinna *et al.* 2005; Ben Chaim *et al.* 2006; Ohana *et al.* 2006; Sahlholm *et al.* 2008; Liu *et al.* 2009) and *Xenopus* LPA (this study). Noticeably, all these receptors have multiple clusters of positively charged residues, mainly arginine and lysine, which resemble the voltage sensor in voltage-dependent ion channels (Bezanilla, 2000). Although these residues are located in the second and third intracellular loops of the GPCR structure, rather than within transmembrane regions that contain the main voltage sensors in other proteins, it is still possible they are involved in voltage sensing (Ben Chaim *et al.* 2006; Mahaut-Smith *et al.* 2008).

The present study not only provides evidence that changes in membrane potential can regulate Ca^{2+} signalling via LPA-receptors, but also indicates conditions under which a change in membrane voltage may exert its most significant effect on signalling via this class of GPCR. Firstly, experiments in Ca^{2+}-free medium showed that the greatest potentiating effect of voltage is observed at the lowest effective agonist concentrations. This characteristic is shared by purinergic $P2Y_1$ receptors (Gurung *et al.* 2008) and can be explained by an action of voltage on the ligand-bound, inactive or partially activated form of the receptor (Gurung *et al.* 2008). Previous work in oocytes has shown that a certain threshold amount of $InsP_3$ is required to generate a detectable response and that this level of $InsP_3$ can be achieved by facilitation between two temporally separated subthreshold $InsP_3$ events (Parker & Miledi, 1989). In the present study, a subthreshold LPA concentration and a depolarizing voltage step may act in a similar cooperative manner since both stimulate release of $InsP_3$. Secondly, at a constant concentration of LPA in the presence of external Ca^{2+}, the depolarization-evoked Ca^{2+} response decreased in magnitude as the agonist-evoked Ca^{2+} response increased (see supplementary Fig. 1 showing this clear inverse correlation). Since the response to depolarization became a net decrease in intracellular Ca^{2+} for oocytes displaying

the largest responses to agonist, the most likely explanation for this trend is a shift in balance from predominantly Ca^{2+} release for low LPA-evoked responses towards a greater contribution from Ca^{2+} influx as the agonist response increases (Putney, 1986). Thus, for the larger responses, the dominant effect of depolarization becomes a reduction in inward driving force for Ca^{2+} influx through store-operated and other pathways.

Given the widespread presence of LPA-receptors in adult and developing tissues (Ishii *et al.* 2004; Noguchi *et al.* 2009), this effect of membrane potential represents a potentially important mechanism whereby LPA responses can be modified by electrogenic influences. Depolarizing steps are also able to induce Ca^{2+} responses in the presence of a subthreshold agonist concentration (Fig. 3*A*) and, therefore, voltage control of GPCRs can be considered as a mechanism of coincidence detection between chemical signals and electrical stimuli. Consequently, in cellular structures such as dendrites, where low levels of neuro-transmitters acting on GPCRs are released and changes in membrane potential are constantly occurring in the form of post-synaptic potentials and backpropagated action potentials, the voltage dependence of GPCR-mediated Ca^{2+} responses could play a major role in signalling.

In contrast to the clear bipolar nature of the voltage dependence of Ca^{2+} release during stimulation of muscarinic and $P2Y_1$ receptors in mammalian cells (Ganitkevich & Isenberg, 1993; Mahaut-Smith *et al.* 1999; Martinez-Pinna *et al.* 2004), only depolarizing voltage steps could be shown to have a marked effect during LPA stimulation in the present study (Fig. 1*D* and *E*). The lack of a clear effect of hyperpolarizing voltage steps is most likely due to the transient nature of the Ca^{2+} response to LPA and the slow speed of the voltage-dependent response in oocytes compared to previous studies. However, we cannot rule out that differences in the structures of GPCRs account for their relative sensitivity to hyper-polarizing voltage steps. A further difference between the effect of voltage on $P2Y_1$ receptors in megakaryocytes compared to LPA receptors in *Xenopus* oocytes was the influence of the holding potential on the initial agonist-evoked $[Ca^{2+}]_i$ response. The peak response to LPA was accelerated and enhanced in a graded manner as the holding potential was shifted from -80 mV to -20 mV whereas $P2Y_1$ receptor responses to 1 μM ADP were reduced in amplitude by a depolarizing shift in holding potential (Gurung *et al.* 2008). At present it is unclear precisely why LPA receptor responses are affected by holding voltage and $P2Y_1$ receptors are not. However, the most likely explanation is that the Ca^{2+} content of the megakaryocyte stores is more susceptible to short periods of reduced Ca^{2+} influx that result from depolarization, together with the slower nature of the Ca^{2+} increases in the oocytes. The latter could allow a tonic effect of voltage on the initial agonist-evoked Ca^{2+} response to be unmasked.

Despite a definitive answer to this issue, the ability of a constant holding voltage to modulate responses through a $G\alpha_q$-coupled GPCRs significantly extends the extent to which membrane potential can influence signalling through this important family of surface proteins.

A further important consideration when comparing the present results with previous studies of GPCR voltage dependence is cell geometry. Oocytes have a diameter of ∼1 mm and hence the ratio of plasma membrane surface to cytoplasmic volume is reduced compared to other cell types. This could explain the low relative speed of the $[Ca^{2+}]_i$ changes recorded in oocytes. Another morphological feature of the oocyte is the numerous microvilli within the plasma membrane, which significantly increase the cell membrane surface, as indicated by the high apparent specific membrane capacitance ($5 \, \mu F \, cm^{-2}$) of these cells (Zhang & Hamill, 2000). These infoldings could increase the ratio of receptors to sub-plasma-membrane $InsP_3$ receptors and thereby contribute to the robust nature of the depolarization on $[Ca^{2+}]_i$ during LPA exposure in oocytes. Interestingly, in megakaryocytes, where the demarcation membrane system extends the surface plasma membrane throughout the extranuclear volume of the cell, the specific membrane capacitance is also high (average $8 \, \mu F \, cm^{-2}$) and we have previously speculated that this may account for the extremely robust nature of the voltage dependence of $G\alpha_q$-coupled receptor Ca^{2+} signals by increasing the ratio of receptors to available $InsP_3$-dependent Ca^{2+} release sites (Mahaut-Smith *et al.* 1999, 2003, 2008).

In conclusion, we have shown that stimulation of metabotropic LPA receptors in *Xenopus* oocytes confers a voltage sensitivity to the $InsP_3$-dependent Ca^{2+} release from the intracellular stores. This influence is present over a wide range of LPA concentrations with the greatest effect of depolarizing voltage steps at near-threshold levels of agonist. The level of the membrane potential is also able to modulate the initial LPA-evoked Ca^{2+} release in a graded manner. Our results suggest that the principal voltage sensor lies within the receptor itself, rather than a downstream pathway. Given that LPA has widespread biological actions, voltage modulation of LPA signalling arises as an important mechanism for modulation of Ca^{2+} signals both in excitable and non-excitable cells.

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Author contributions

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