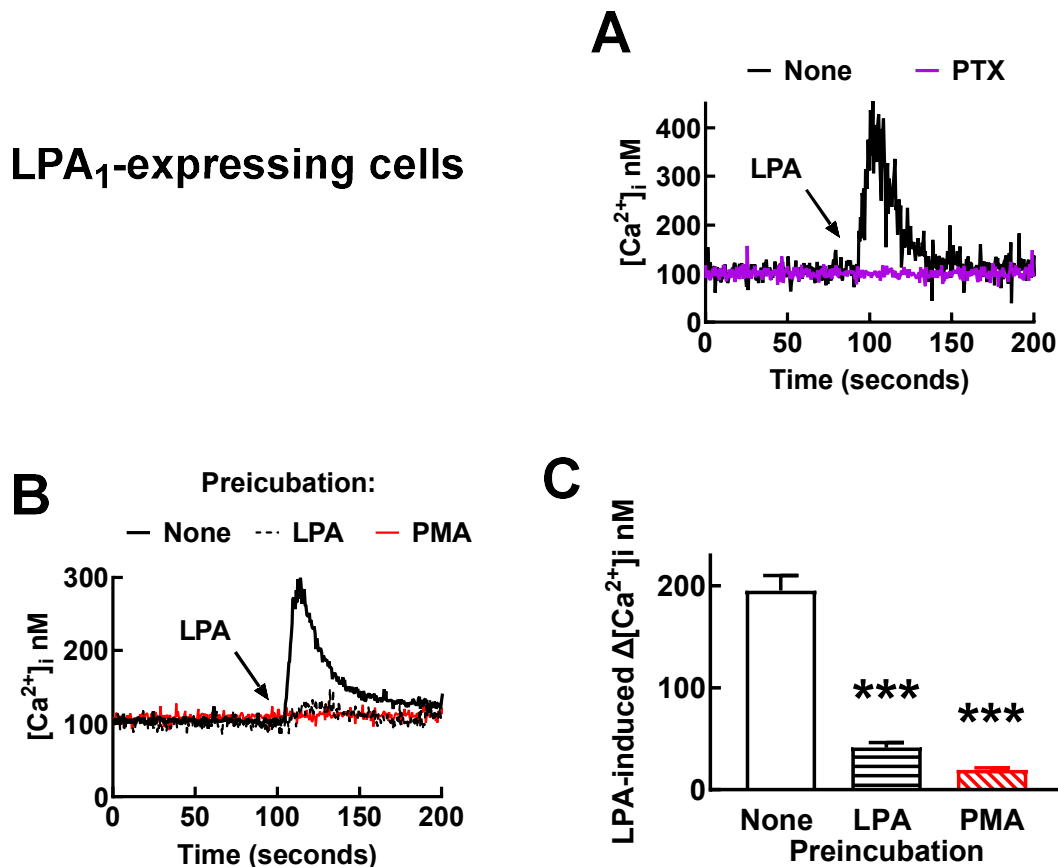


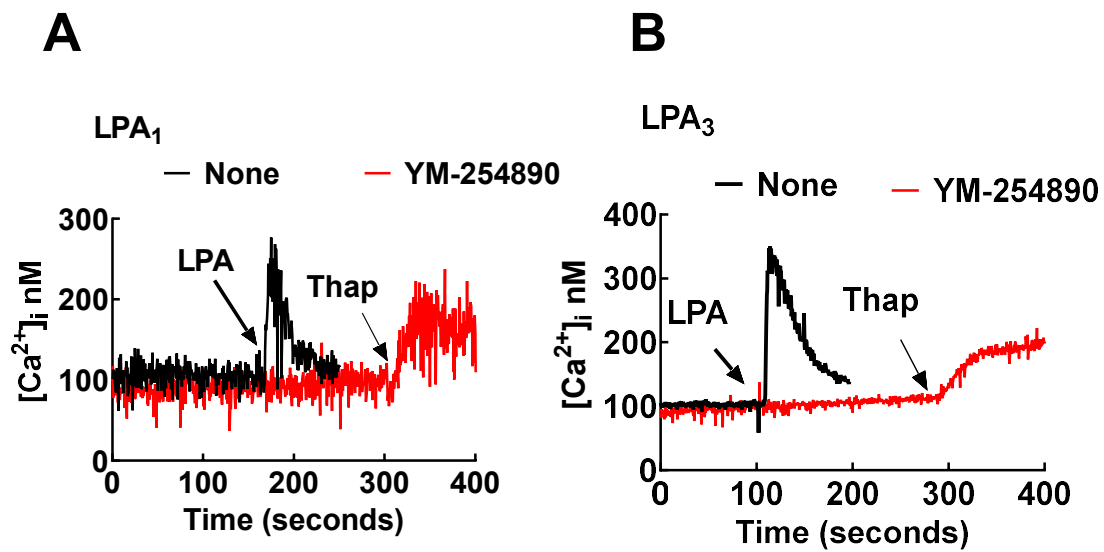
SUPPLEMENTARY MATERIALS

Supplementary Fig. S1. The LPA₁ receptor is functional and promotes heterologous and homologous desensitization. **Panel A**, calcium tracings of cells expressing LPA₁ receptors pre-incubated overnight without (black line) or with pertussis toxin (purple line); arrow indicates the addition of 1 μ M LPA. **Panel B**, calcium tracings in response to 1 μ M LPA of cells pre-incubated without any agent (black line) or with 1 μ M LPA for 5 min and washed before re-challenging (LPA, black dotted line) or with 1 μ M PMA (PMA, red line). **Panel C**, calcium increases in response to LPA of cells pre-incubated without any agent (None, open bar), with 1 μ M LPA for 5 min and washed before re-challenging (LPA, dashed bar) or with 1 μ M PMA (PMA, red dashed bar). The means are plotted, and vertical lines indicate the SEM of 4-5 experiments performed on different days and cell cultures. *** $p < 0.001$ vs. baseline.

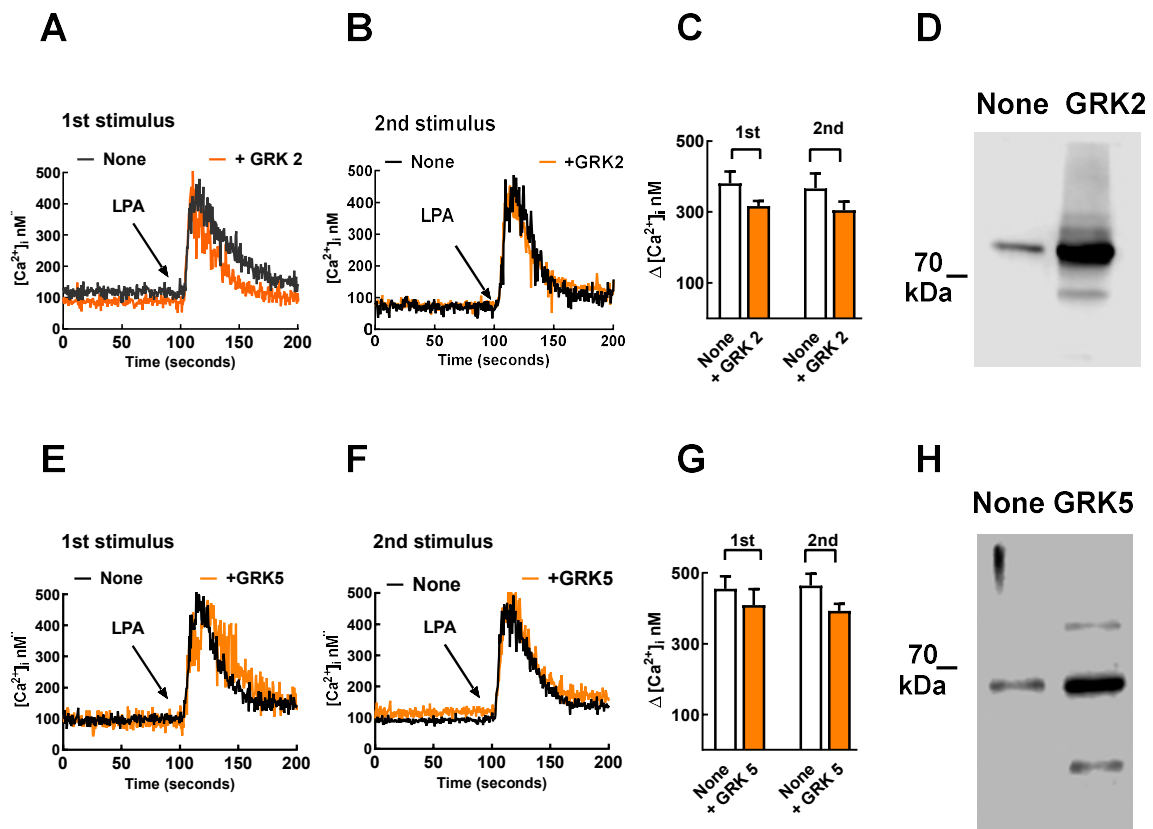
LPA₁-expressing cells



Supplementary Fig. S2. Effect of the $G\alpha_q$ inhibitor, YM-254890, on LPA-induced increase in intracellular calcium concentrations in cells expressing LPA_1 or LPA_3 receptors. Representative calcium tracings of cells expressing LPA_1 (**panel A**) or LPA_3 (**panel B**) receptors were pretreated for 1 min without any agent (black lines) or with 1 μ M YM-254890 (red lines) and challenged with 1 μ M LPA (arrow). When no response was observed, thapsigargin (1 μ M) was added to define the ability of the cells to increase the intracellular calcium concentration.

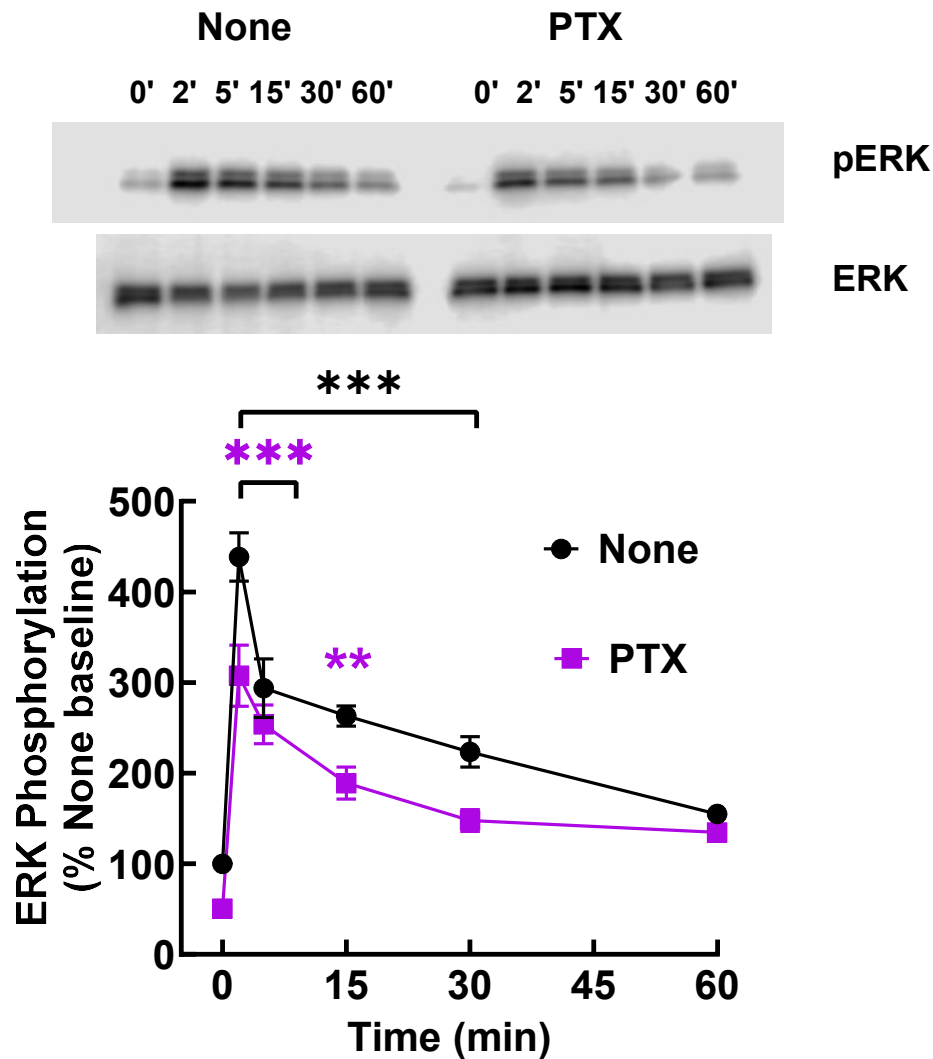


Supplementary Fig. S3. Absence of effect of overexpression of GRK2 and GRK5 on homologous LPA₃ desensitization. Cells expressing the LPA₃ receptor were not transfected (none) or transfected with plasmids for expression of GRK2 (**panels A-D**) or GRK 5 (**panels E-H**). Cells were challenged with 1 μ M LPA (1st stimulus, representative tracings in **panels A and E**), washed, and challenged again with 1 μ M LPA (2nd stimulus, representative tracings in **panels B and F**). LPA-induced increases in intracellular calcium are presented in **panels C and G** (the means are plotted, and vertical lines indicate the SEM of 5-7 experiments performed using different cell cultures). In **panels D** (GRK2, Mr $\approx$ 80 kDa) **and H** (GRK5, Mr $\approx$ 65 kDa) representative Western blots, using GRK2- or GRK5-specific primary antibodies, of cell lysates are presented.

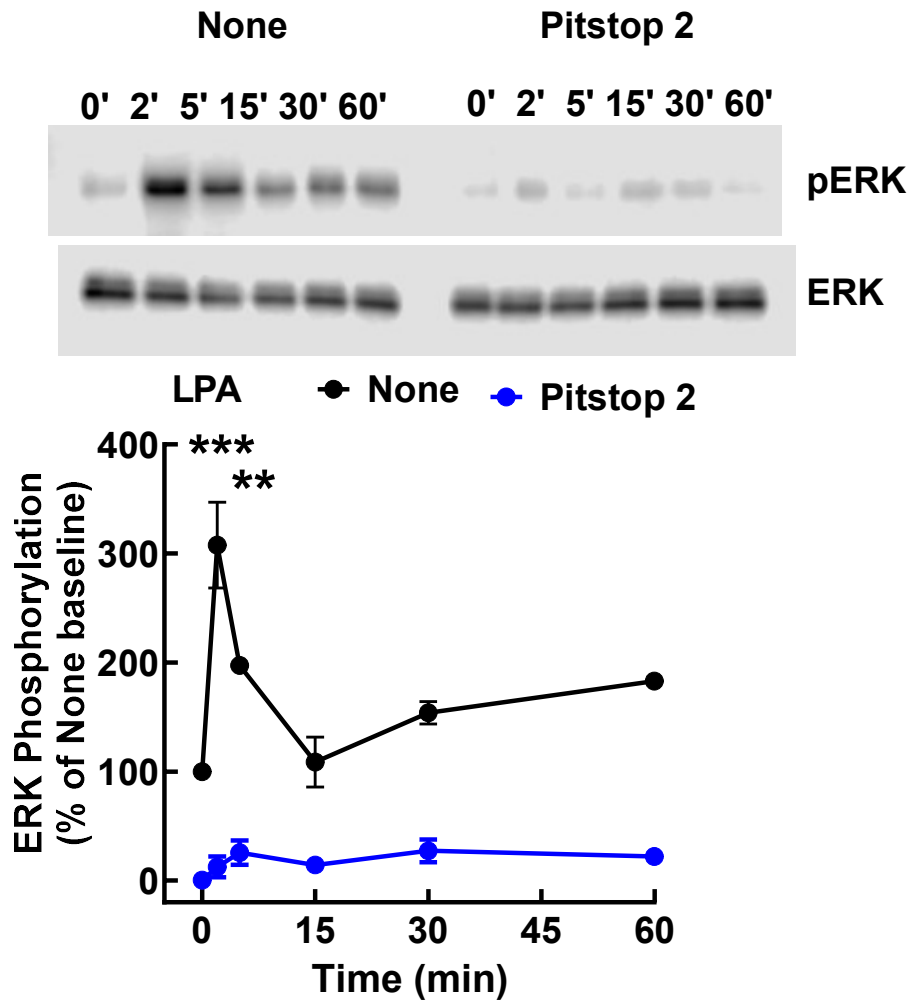


Supplementary Fig. S4. Effect of pertussis toxin on LPA₁-induced ERK 1/2

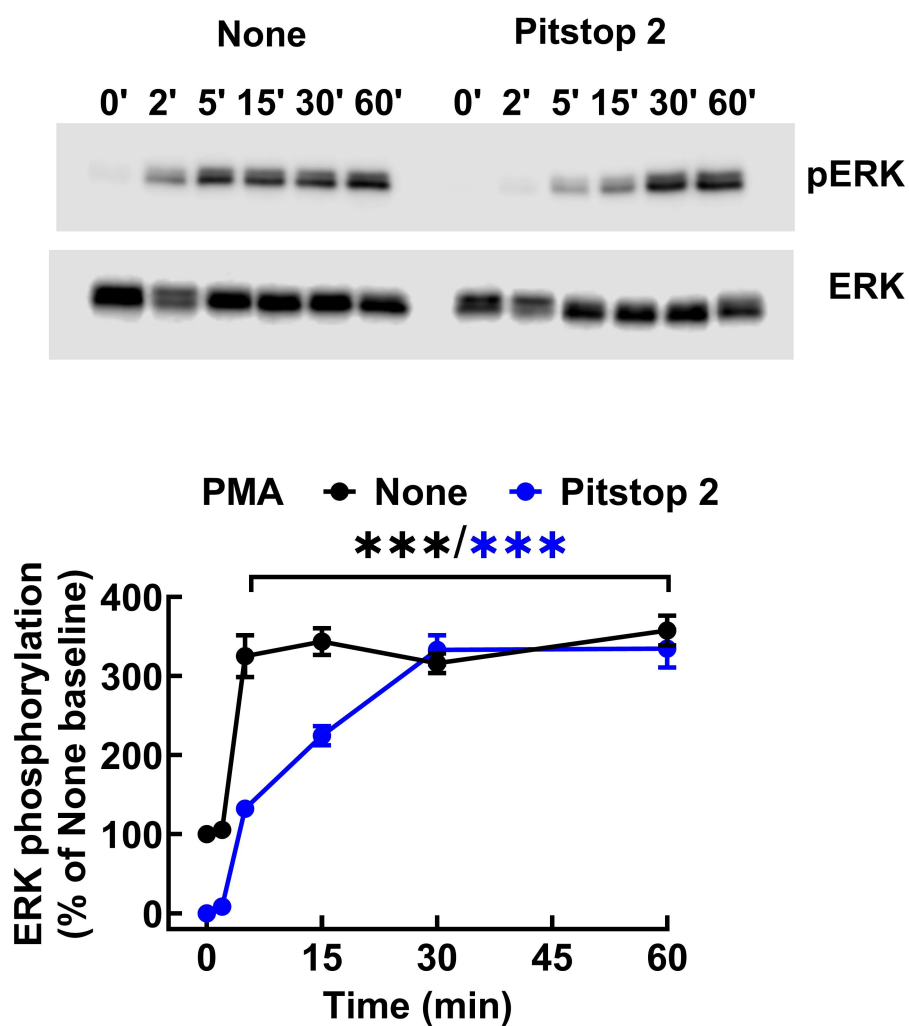
phosphorylation. Cells were incubated overnight with (PTX, purple line) or without (None, black line) pertussis toxin, and cells were challenged with 1 μ M LPA for the times indicated. Phospho-ERK (pERK) and total ERK (ERK) were determined by Western blotting. The means are plotted, and vertical lines indicate the SEM of 4-5 experiments performed on different days using different cell cultures. *** $p < 0.001$, and ** $p < 0.005$ vs. baseline value. Baseline ERK was significantly diminished by pertussis toxin treatment, $p < 0.001$. Representative Western blots are presented above the figure.



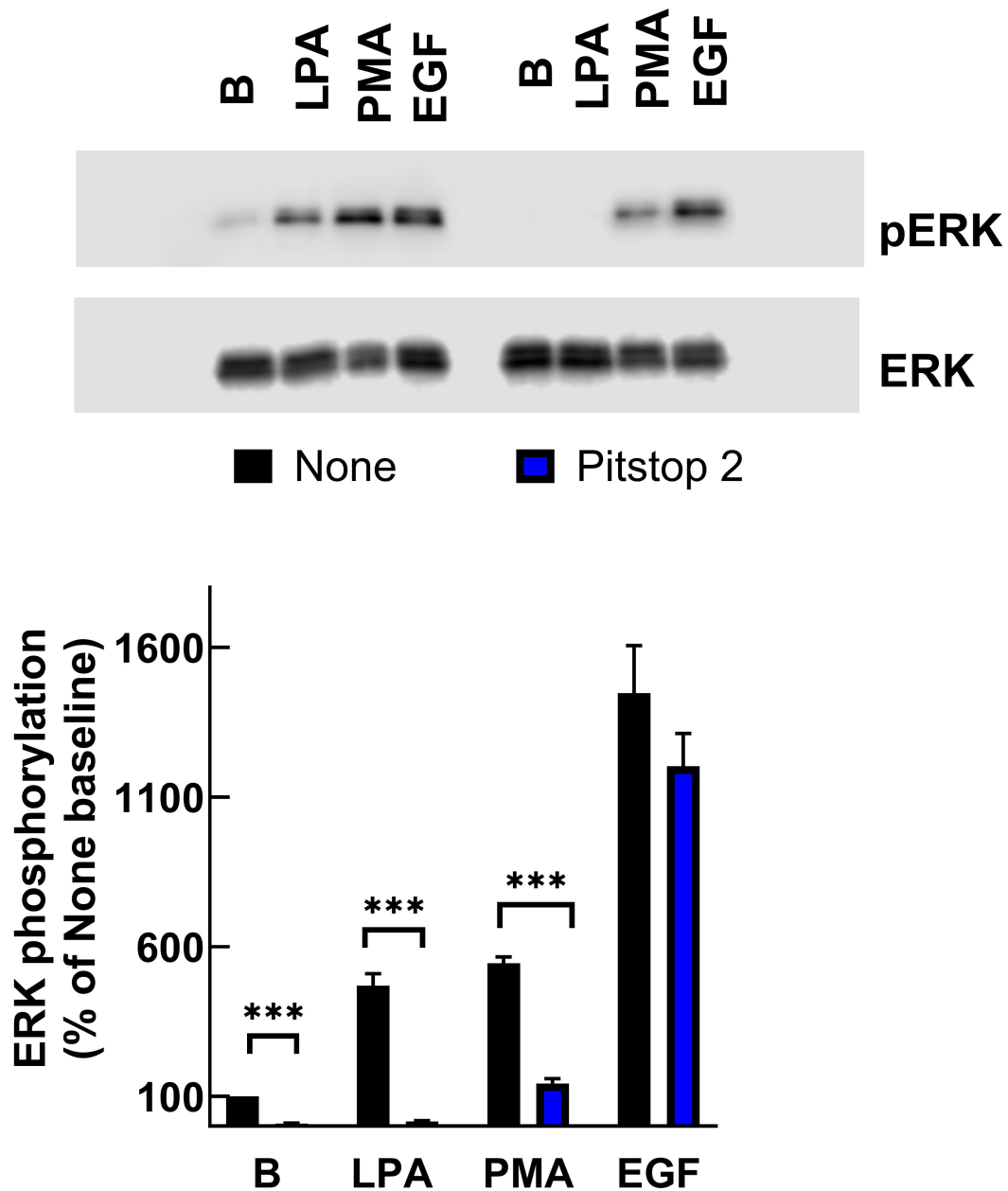
Supplementary Fig. S5. Effect of Pitstop 2 on LPA-induced ERK phosphorylation. LPA₃-expressing cells were pre-incubated without (None) or with 10 μ M Pitstop 2 for 15 min and then treated with 1 μ M LPA for the times indicated. The means are plotted, and vertical lines indicate the SEM of 4-5 experiments performed on different days using distinct cell cultures. ***p < 0.001, **p < 0.005 vs. baseline value. The clathrin inhibitor, Pitstop 2, decreased baseline ERK 1/2 phosphorylation, p < 0.001. Representative Western blots are presented above the figure.



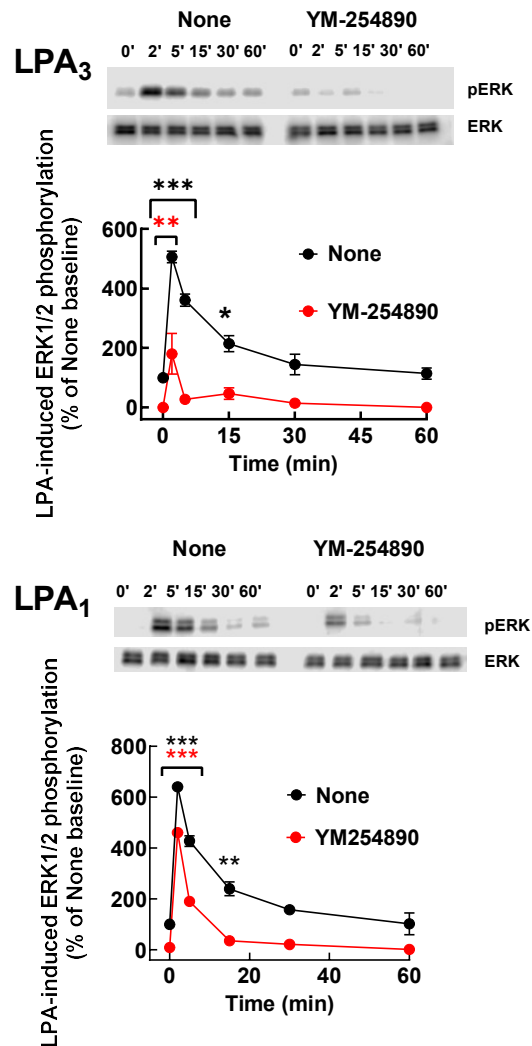
Supplementary Fig. S6. Effect of Pitstop 2 on PMA-induced ERK phosphorylation. LPA₃-expressing cells were pre-incubated without (None) or with 10 μ M Pitstop 2 for 15 min and then treated with 1 μ M PMA for the times indicated. The means are plotted, and vertical lines indicate the SEM of 4-5 experiments performed on different days using distinct cell cultures. ***p < 0.001 vs. baseline value. The clathrin inhibitor, Pitstop 2, decreased baseline ERK 1/2 phosphorylation, p < 0.001. Representative Western blots are presented above the figure.



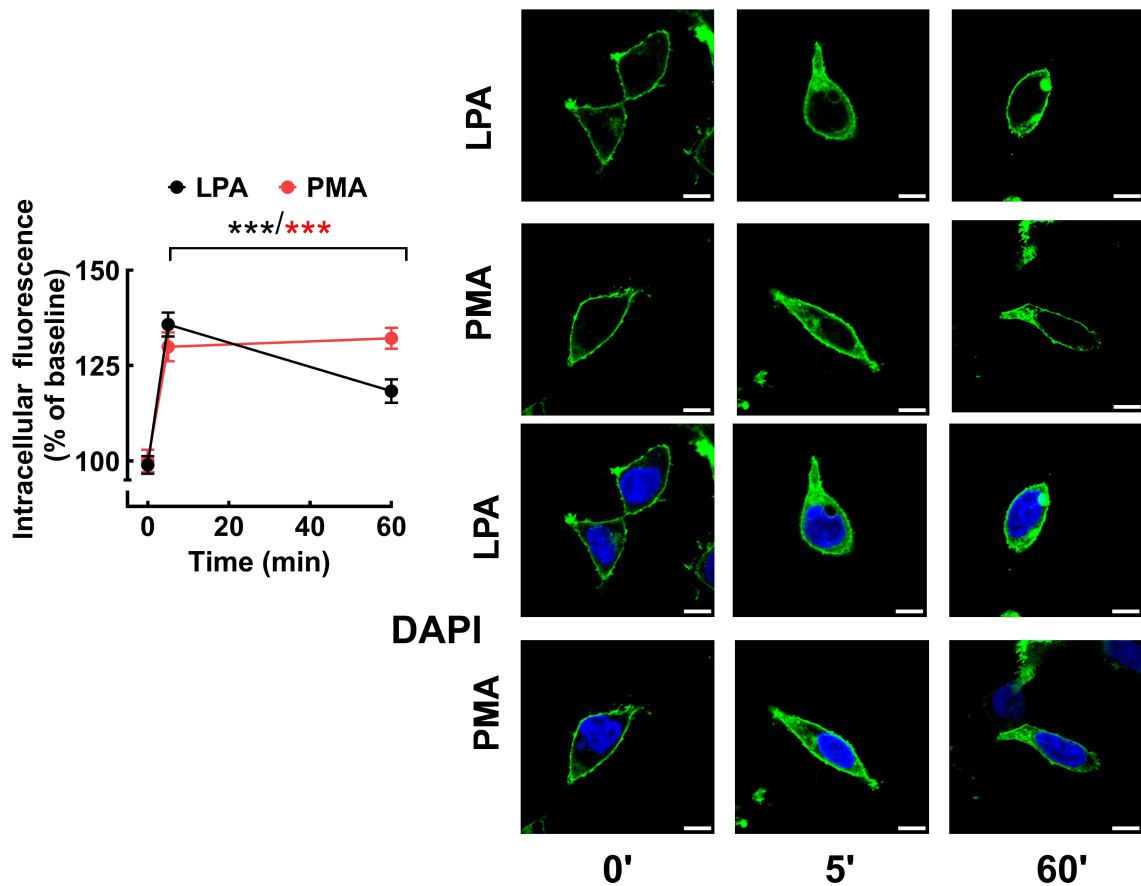
Supplementary Fig. S7. Effect of Pitstop 2 LPA-, PMA- and EGF-mediated ERK phosphorylation. LPA₃-expressing cells were pre-incubated without (None) or with 10 μ M Pitstop 2 for 15 min and then treated with 1 μ M LPA, 1 μ M PMA, or 100 ng/ml EGF for 15 min. *** $p < 0.001$. Representative Western blots are presented above the figure.



Supplementary Fig. S8. Effect of the $G\alpha_q$ inhibitor, YM-254890, on LPA-induced ERK phosphorylation in LPA₁ and LPA₃-expressing cells. LPA₃- (panel A) or LPA₁- (panel B) expressing cells were pre-incubated for 1 min without (None, black lines and symbols) or 1 μ M YM-254890 (red lines and symbols) and then incubated with 1 μ M LPA for the times indicated. *** $p < 0.001$, and * $p \leq 0.05$ vs. their respective baseline values. YM-254890, decreased baseline ERK 1/2 phosphorylation, $p < 0.001$. Representative Western blots are presented above the figures.

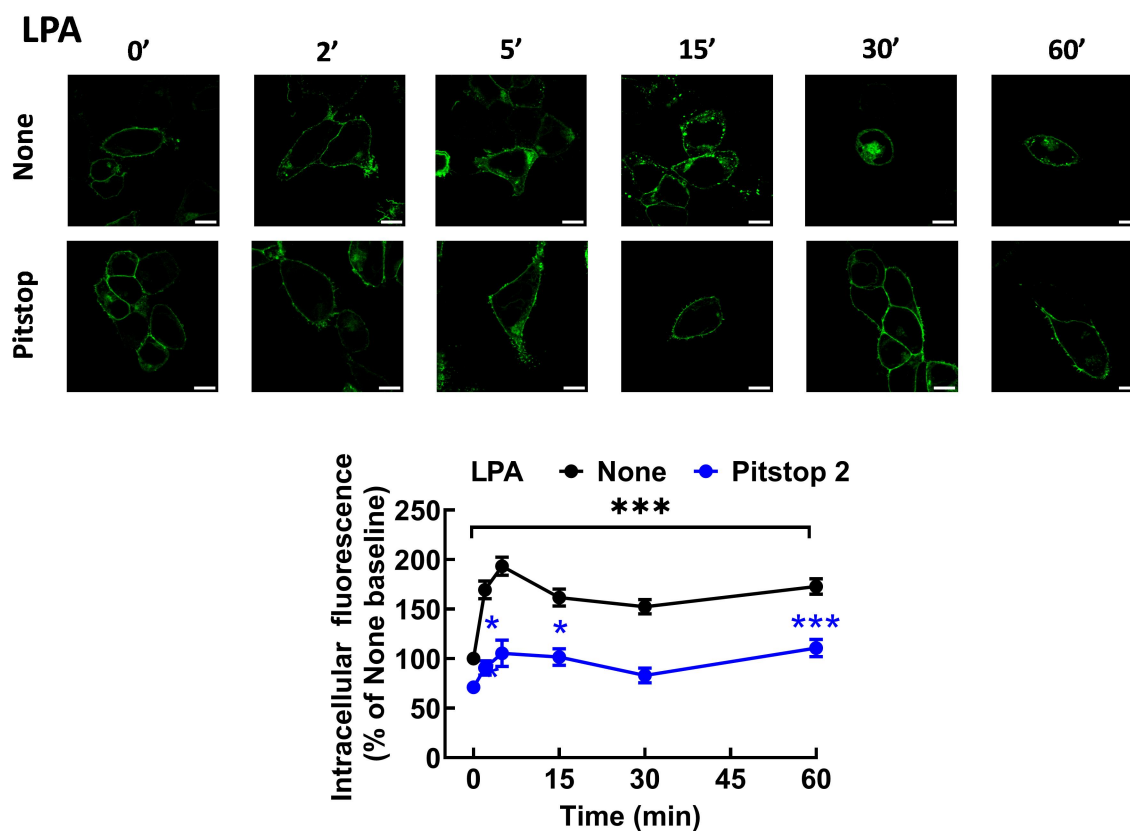


Supplementary Fig. S9. Receptor internalization in cells in which receptor expression was induced by a reduced (1 ng/ml) concentration of doxycycline. Cells were incubated for the times indicated in the presence of 1 μ M LPA or 1 μ M PMA. Internalization is presented as the percentage of baseline intracellular fluorescence. The mean is plotted, and vertical lines indicate the SEM of 14 images obtained from each of 5 experiments performed on different days. At the right side of the graph, representative images without or with the nuclei stained with DAPI are presented. Scale bars 10 μ m. *** p < 0.001 vs baseline (0').



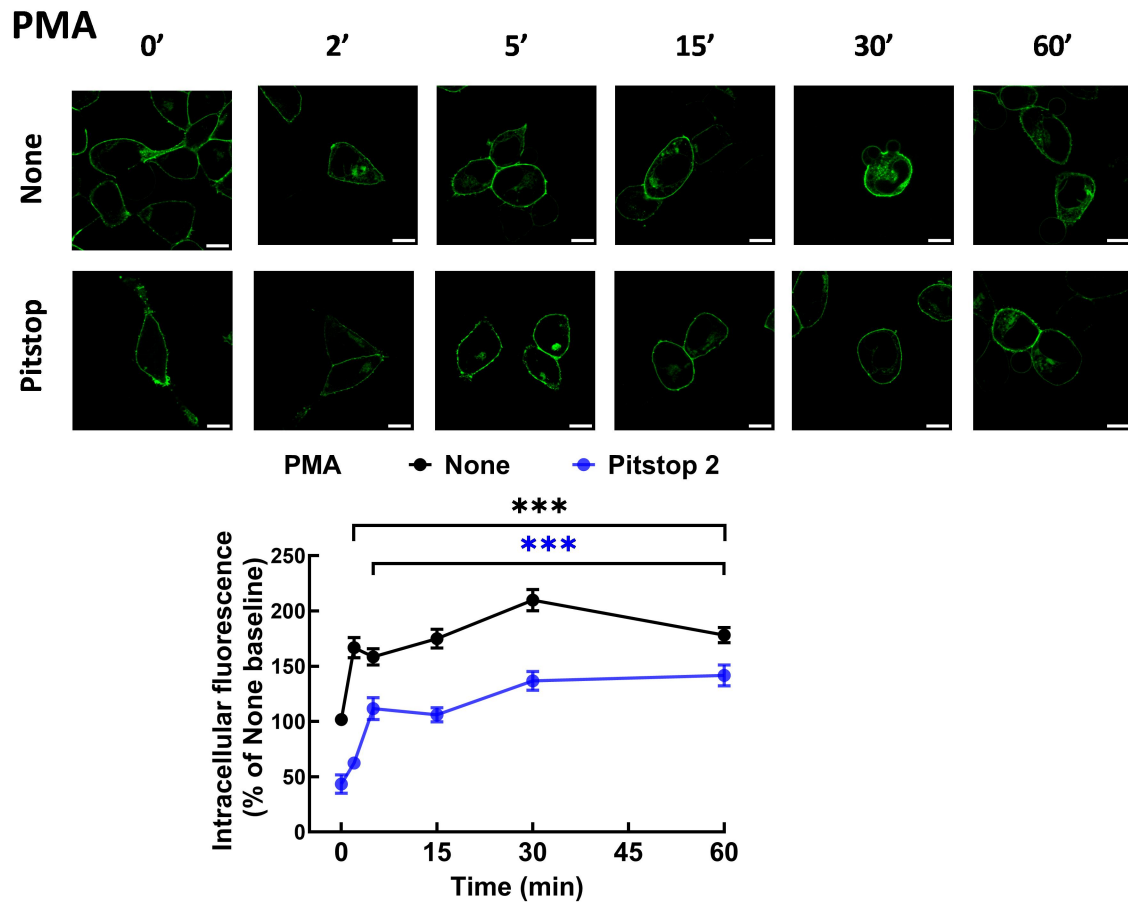
Supplementary Fig. S10. Effect of Pitstop 2 on LPA-induced LPA₃ internalization.

Cells expressing the LPA₃ receptor were pre-incubated without any agent (None, black line and symbols) or with Pitstop 2 (red line and symbols) for 15 min and then stimulated with 1 μ M LPA. Pitstop 2 decreased baseline internal fluorescence, *** $p < 0.001$. Baseline internal fluorescence was decreased by pre-incubation with Pitstop 2 ($p < 0.001$). When compared to their baseline, LPA increased internalization in the presence of Pitstop 2 (blue asterisks) *** $p < 0.001$, ** $p < 0.005$, and * $p < 0.05$ vs. baseline value. Representative images are presented above the figure. Scale bars 10 μ m.



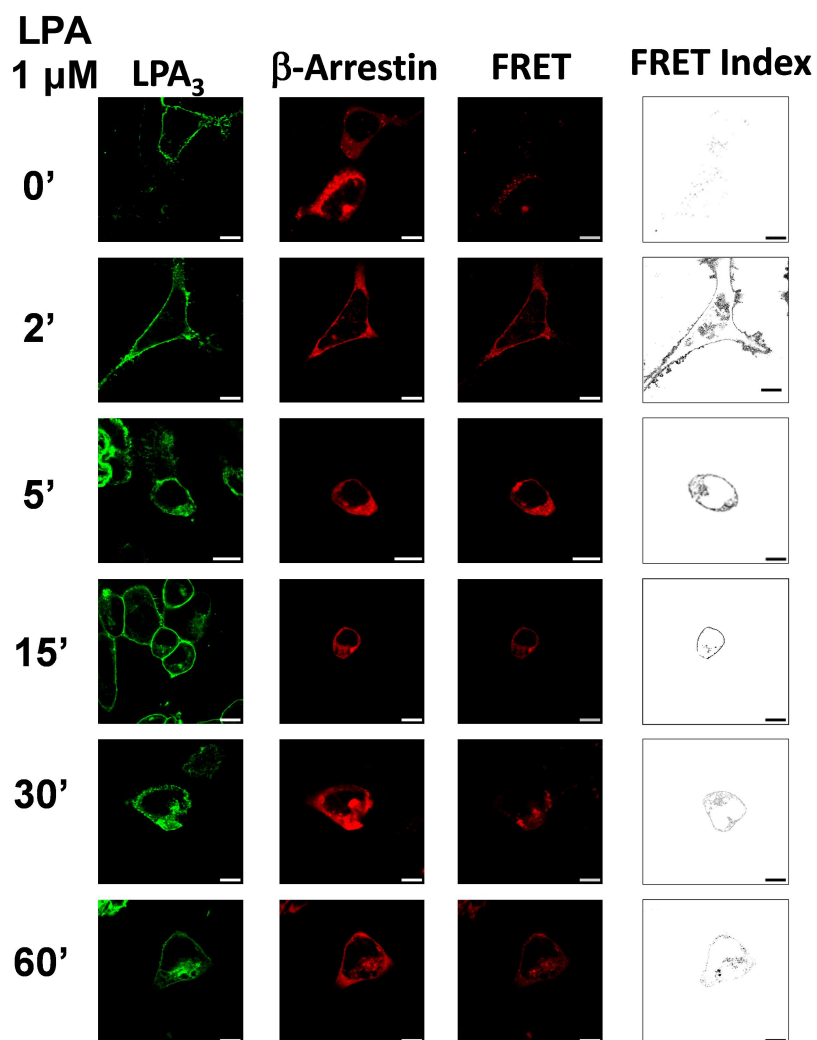
Supplementary Fig. S11. Effect of Pitstop 2 on PMA-induced LPA₃ internalization.

Cells expressing the LPA₃ receptor were pre-incubated without any agent (None, black line and symbols) or with Pitstop 2 (red line and symbols) for 15 min and then stimulated with 1 μ M PMA. ***p < 0.001 vs. none baseline, (blue asterisks) ***p < 0.001 vs. baseline (Pitstop 2). Baseline internal fluorescence was decreased by pre-incubation with Pitstop 2 (p < 0.001). Representative images are presented above the figure. Scale bars 10 μ m.

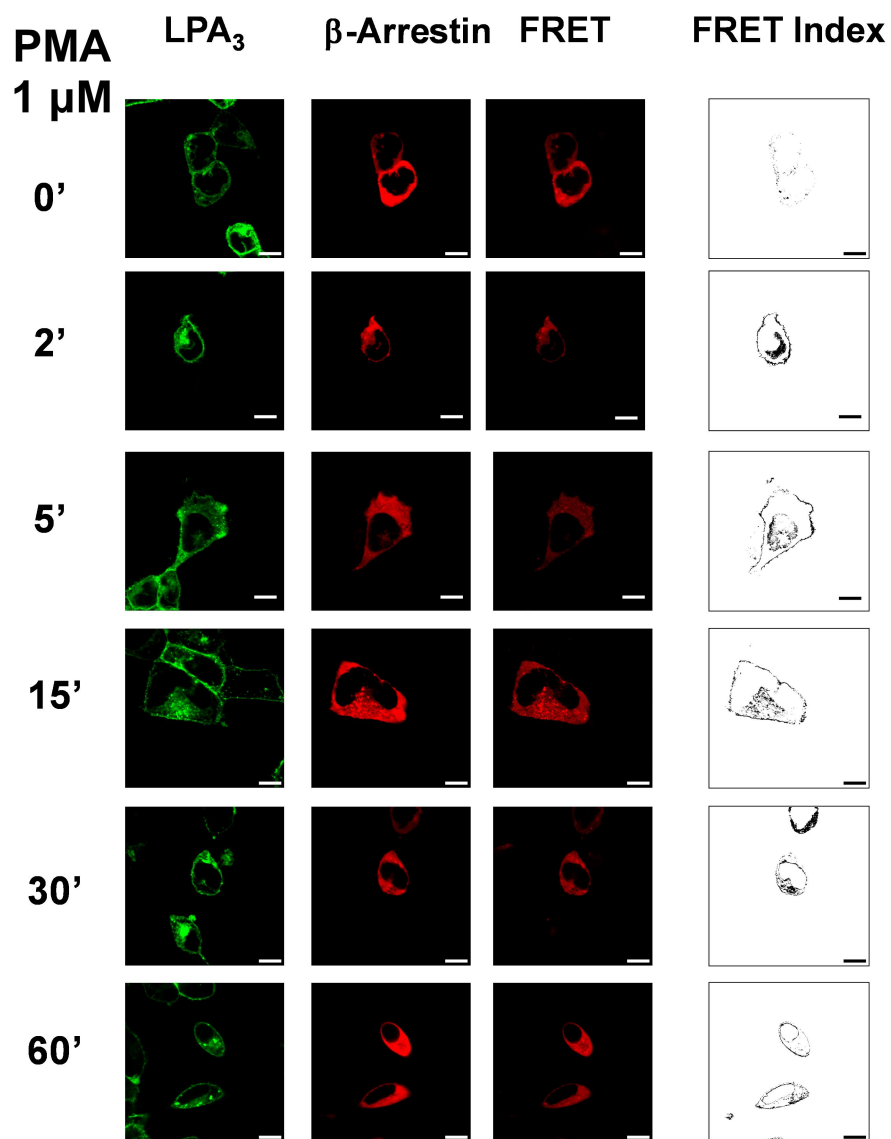


Supplementary Fig. S12. Representative images of the time-course of the effect of LPA on eGFP-tagged LPA₃ receptor-mCherry-tagged β -arrestin 2 interaction (FRET).

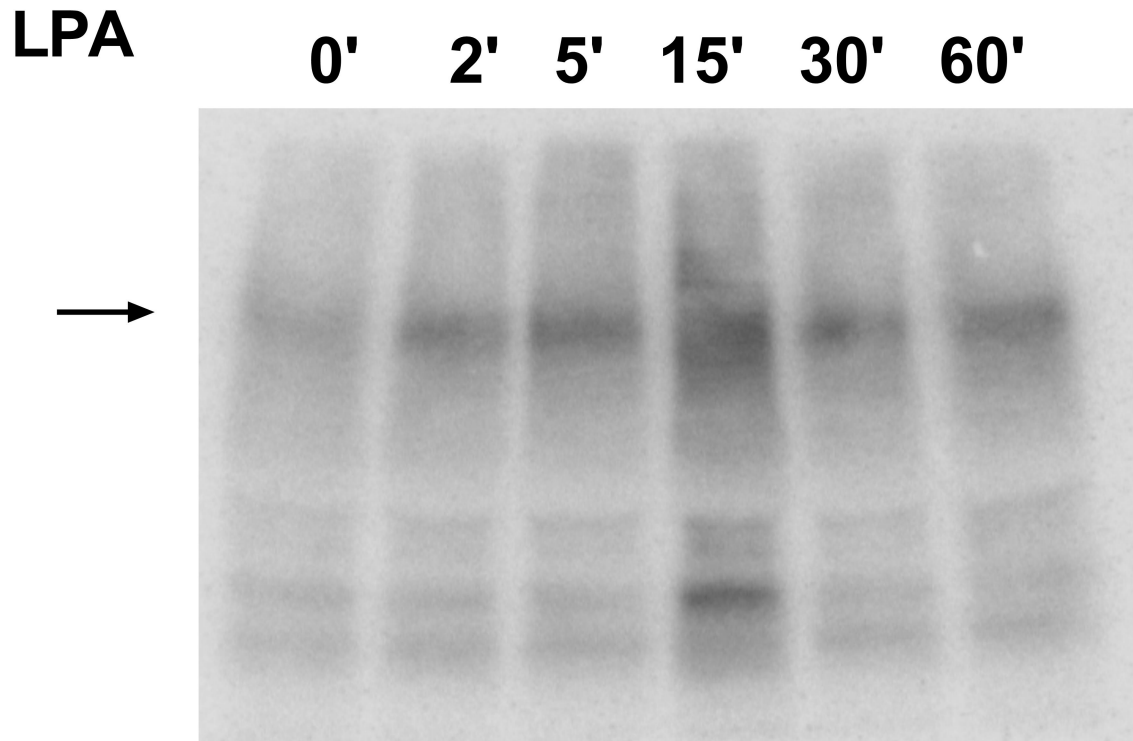
Cells were incubated for the times indicated in the presence of 1 μ M LPA. The following images are presented: LPA₃-eGFP fluorescence (eGFP was excited and its fluorescence was recorded; first column), β -arrestin-mCherry fluorescence (mCherry was excited and its fluorescence was recorded; second column), FRET (raw FRET) (eGFP was excited, the laser to excite mCherry remained off, and mCherry fluorescence was recorded; third column) and “FRET index” image (images processed with the ImageJ "FRET and Colocalization Analyzer", fourth column). Scale bars: 10 μ m.



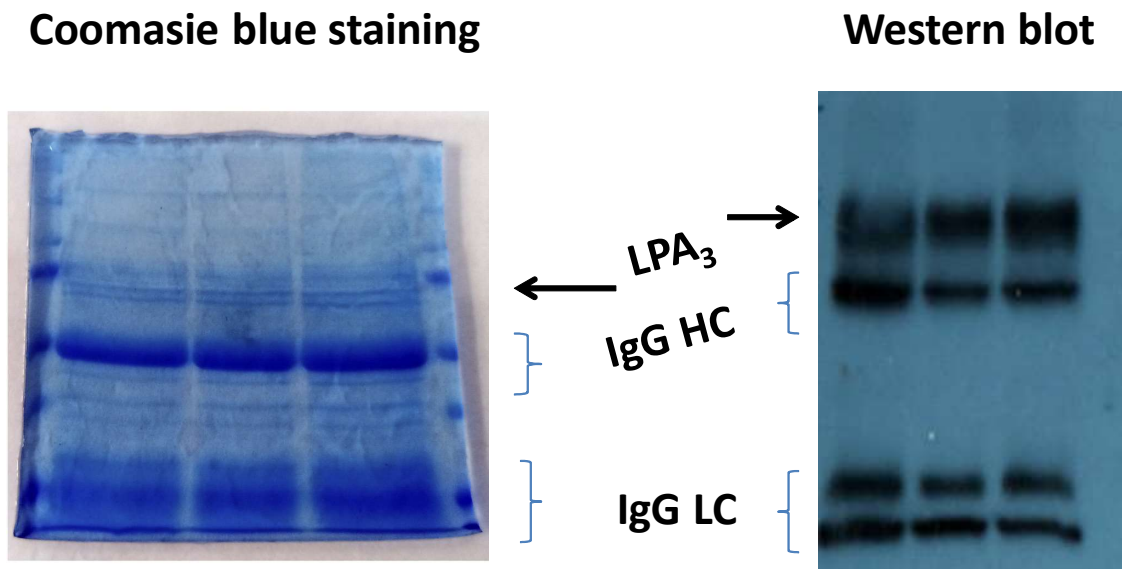
Supplementary Fig. S13. Representative images of the time-course of the effect of PMA on eGFP-tagged LPA₃ receptor-mCherry-tagged β -arrestin 2 interaction (FRET). Cells were incubated for the times indicated in the presence of 1 μ M PMA. The following images are presented: LPA₃-eGFP fluorescence (eGFP was excited and its fluorescence was recorded; first column), β -arrestin-mCherry fluorescence (mCherry was excited and its fluorescence was recorded; second column), FRET (raw FRET) (eGFP was excited, the laser to excite mCherry remained off, and mCherry fluorescence was recorded; third column) and “FRET index” image (images processed with the ImageJ "FRET and Colocalization Analyzer", fourth column). Scale bars: 10 μ m.



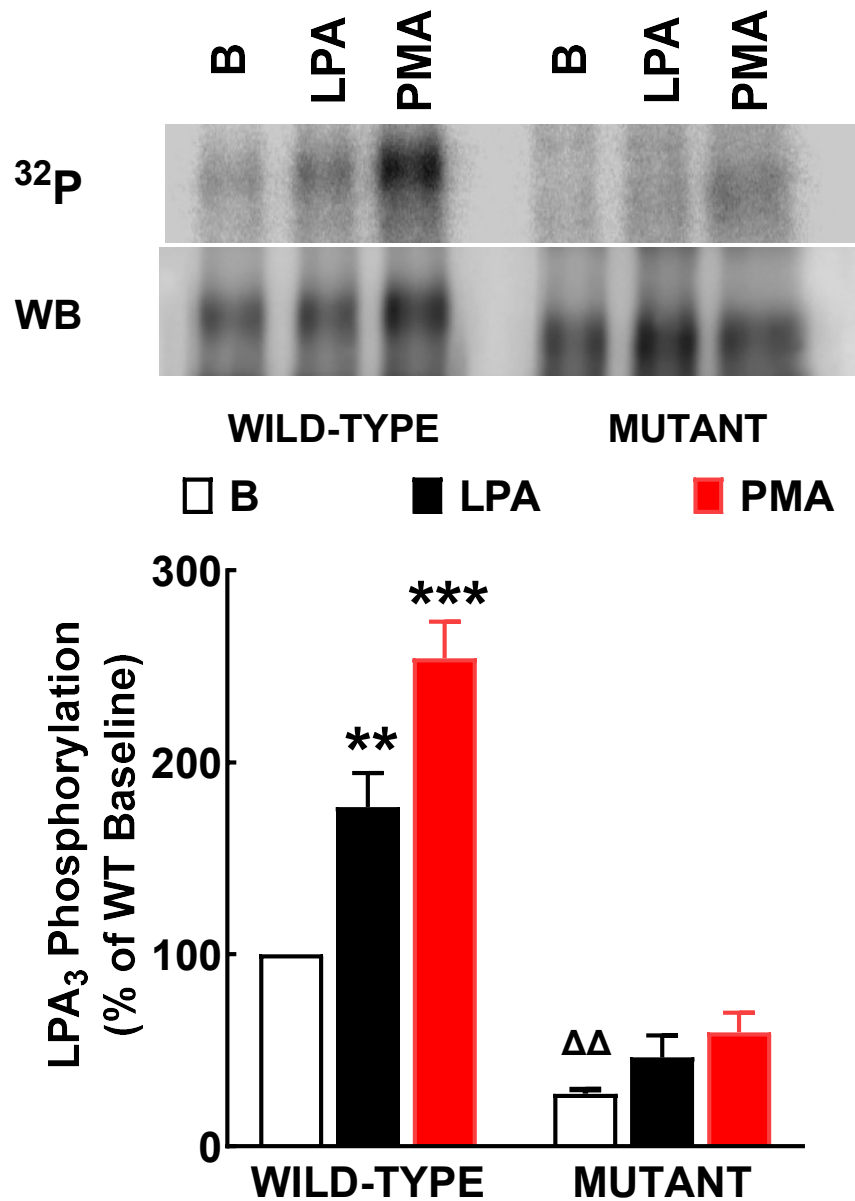
Supplementary Fig. S14. Representative image of the time course of LPA-induced LPA₃ receptor phosphorylation. The image shows a whole membrane autoradiograph of the phosphorylation pattern of the LPA₃ receptor when activated with LPA. The arrow indicates the LPA₃ receptor construct.



Supplementary Fig. S15. Representative LPA₃ immunopurification samples used for mass spectrometry analysis. The image on the left shows an SDS-polyacrylamide gel electrophoresis stained with Coomassie blue; the bands detected at $M_r \approx 70$ kD, corresponding to the LPA₃ construct (arrow), were cut and sent to mass spectrometry analysis. Identification of the LPA₃ receptor-eGFP (arrow) was performed by Western blot (**right panel**). Identification of the IgG heavy (IgG HC) and light (IgG LC) chains are indicated.



Supplementary Fig. S16. LPA- and PMA-induced wild type and Mutant LPA₃ receptor phosphorylation. Cells expressing the wild-type or mutant receptors were challenged with 1 μ M LPA or 1 μ M PMA for 10 min.. Receptor phosphorylation was determined as in Fig. 5 and it is expressed as the percentage of the wild-type baseline value. The means are plotted, and vertical lines indicate the SEM of 4 experiments performed. *** $p < 0.001$, ** $p < 0.01$, $\Delta\Delta p < 0.01$ vs. wild-type baseline value. Representative autoradiographs (³²P) and Western blots (WB) are presented above the graph.



Supplementary Fig. S17. Expression of wild-type and mutant LPA₃ receptors.

Representative images of cells expressing the wild-type (panel A) and the mutant receptors (panel B). Bars, 10 μ m. Panel C shows the fluorescence intracellular/total ratio detected in cells expressing wild-type and mutant receptors. The means are plotted, and vertical lines indicate the SEM of 5 experiments in which 10 cells were analyzed. *** $p < 0.001$, Student t-test. Western blot detection of immunopurified wild-type and mutant LPA₃ receptors. Data were normalized to the abundance detected in the wild-type samples. The means are plotted, and vertical lines indicate the SEM of 4 with samples in triplicate, including variation in the control samples. A representative Western blot is shown above the figure.

