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Figures and figure supplements

PIP_2 depletion promotes TRPV4 channel activity in mouse brain capillary endothelial cells

Osama F Harraz *et al*

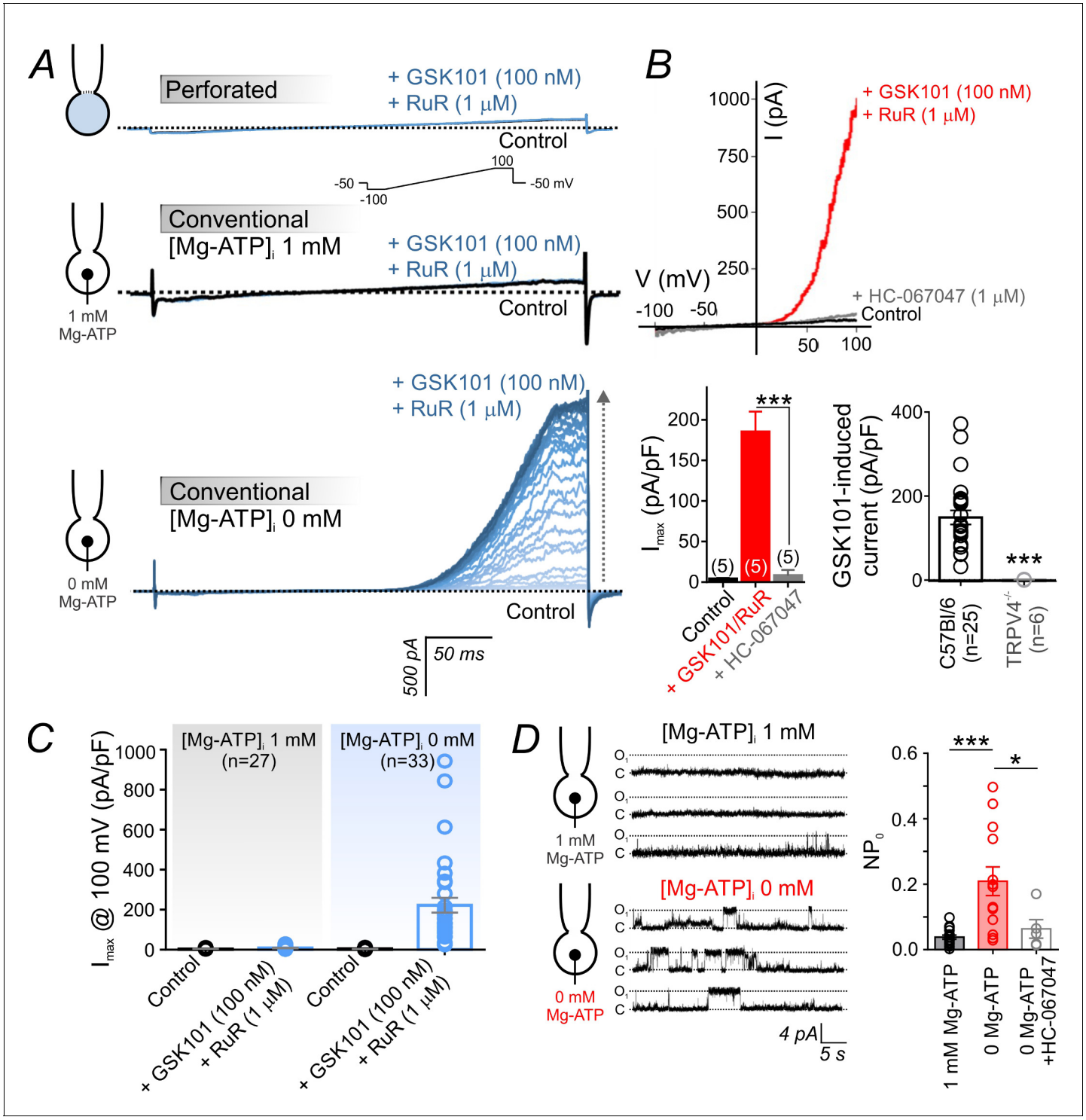

Figure 1. Intracellular ATP suppresses TRPV4 currents in cECs. (**A**) Representative traces of TRPV4 currents recorded from freshly isolated cECs using voltage ramps (−100 to 100 mV, from a holding potential −50 mV; inset) and different patch-clamp configurations before (black) and after (blue) the application of 100 nM GSK101 and 1 μM RuR. *Top*: Currents recorded in the perforated whole-cell configuration. *Middle*: Currents recorded in the conventional whole-cell configuration (dialyzed cytoplasm, 1 mM Mg-ATP in the pipette solution). *Bottom*: Currents recorded in the conventional whole-cell configuration (0 mM Mg-ATP in the pipette solution) developed gradually over ~4 min. (**B**) Current-voltage relationship (*top*) and summary data (*bottom left*) of currents recorded before (control) and after the cumulative application of GSK101 (100 nM)+RuR (1 μM) followed by HC-067047 (1 μM) (means ± SEM, ***p<0.001, unpaired Student's t-test; n = 5 each). *Bottom right*: Individual-value plot of peak outward GSK101 (100 nM)-induced currents in cECs isolated from brains of C57Bl/6 (n = 25) or TRPV4$^{-/-}$ (n = 6) mice. A minimum duration of ~5 min after the application of GSK101 was

Figure 1 continued on next page

Figure 1 continued

allowed for outward TRPV4 current to develop in each cEC. Data are presented as means ± SEM (***p<0.001, unpaired Student's t-test). (**C**) Individual-value plot of peak outward currents recorded at 100 mV before and after the application of GSK101 (100 nM) onto cECs dialyzed with 0 or 1 mM Mg-ATP in the pipette solution. Individual data points are shown together with means (column bars) and SEM (error bars). (**D**) Representative traces (*left*) and summary individual-value plot (*right*) of TRPV4 single-channel activity. Single-channel openings of TRPV4 channels were recorded as outward quantal K^+ currents from cECs in the absence of GSK101 (conventional whole-cell configuration; holding potential, +50 mV; sampling rate, 20 kHz; low-pass filter frequency, 1 kHz; average recording time for each data point, 6 min). cECs were dialyzed with 0 mM (n = 13) or 1 mM (n = 16) Mg-ATP. One group of cECs dialyzed with 0 mM Mg-ATP was treated with 1 μM HC-067047 (n = 5). Data are presented as means (column bars) ± SEM (error bars; *p<0.05, ***p<0.001, one-way ANOVA followed by Tukey's multiple comparisons test).

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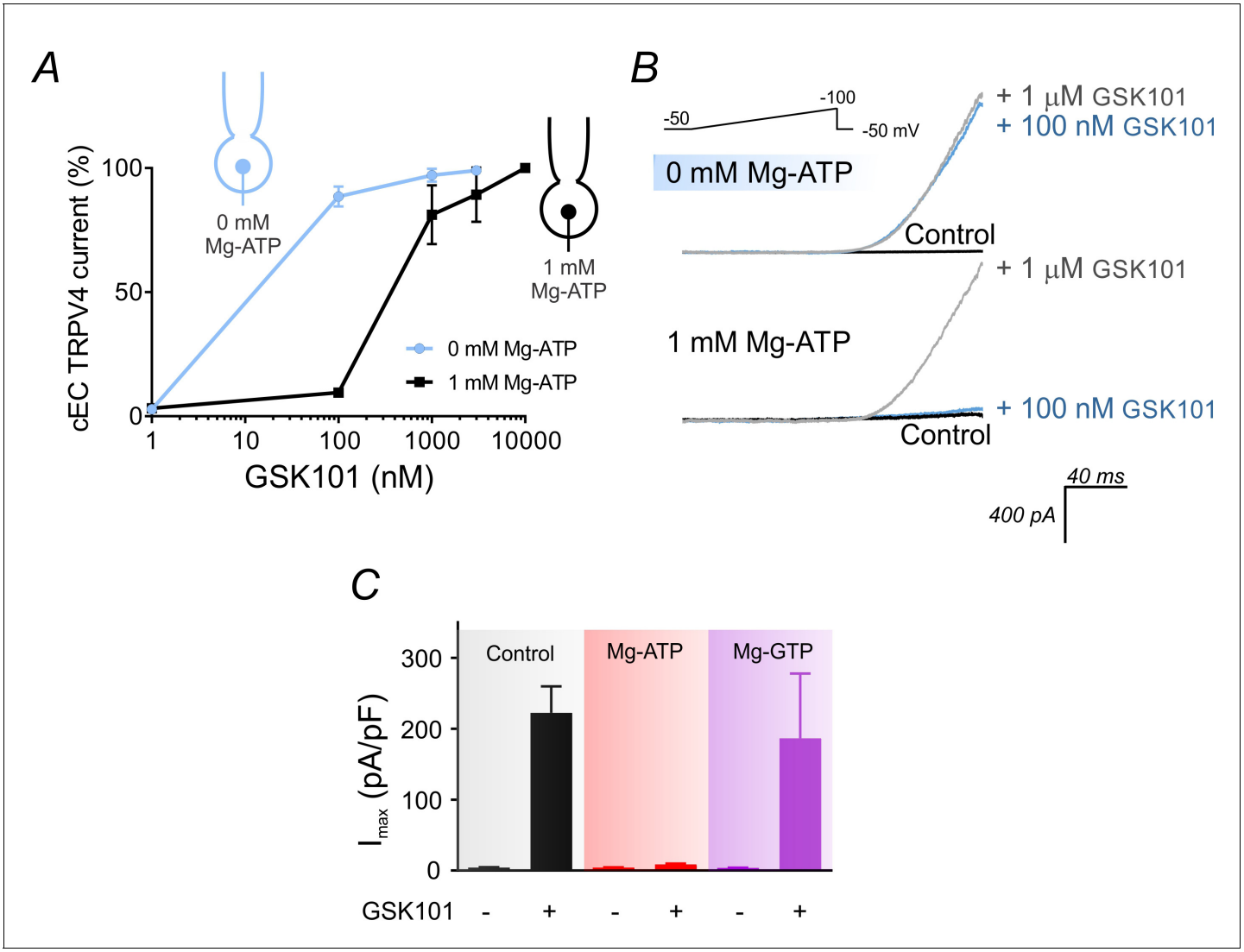

Figure 1—figure supplement 1. Intracellular ATP suppresses TRPV4 activity in cECs. (**A**) GSK101 concentration-response curve. Currents were measured at 100 mV using the conventional whole-cell configuration in cECs in the presence of 1 μM RuR. cECs were dialyzed with 0 or 1 mM Mg-ATP in the pipette solution and GSK101-evoked currents developed over ~5 min. TRPV4 current densities with 100 nM GSK101 were 192 ± 30 pA/pF (0 mM Mg-ATP) and 13 ± 2 pA/pF (1 mM Mg-ATP). Data are presented as means ± SEM (0–1000 nM GSK101, n = 9 cECs; 3–10 μM GSK101, n = 2–3 cECs). (**B**) Representative traces from two cECs dialyzed with 0 mM Mg-ATP (*upper*) or 1 mM Mg-ATP (*lower*). Currents were recorded using voltage ramps (−50 to 100 mV) before and after the application of 100 nM and then 1 μM GSK101. (**C**) Summary data showing outward currents at 100 mV, before and after 100 nM GSK101, recorded in dialyzed cECs using different intracellular nucleotide compositions: no nucleotides (control), 1 mM Mg-ATP, or 1 mM Mg-GTP (n = 7–25). For each cEC, a minimum of 5 min was allowed for outward TRPV4 current to develop after the application of GSK101.

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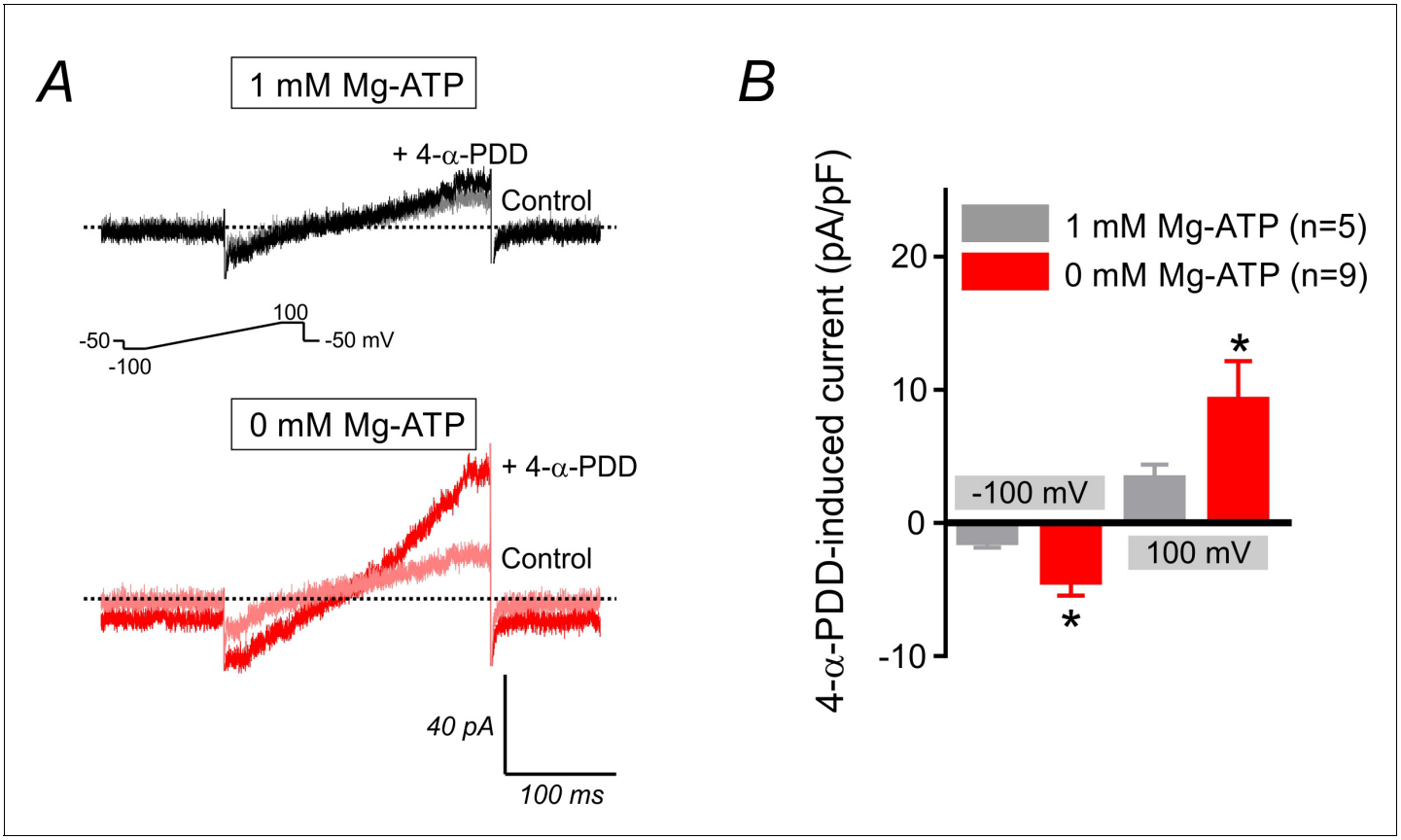

Figure 1—figure supplement 2. 4-α-PDD-induced current in cECs. (**A**) Representative traces recorded from two cECs dialyzed with 1 mM (*upper*) and 0 mM Mg-ATP (*lower*), before and after the application of 4-α-PDD (5 μM), using 300 ms voltage ramps (−100 to 100 mV, inset). Ruthenium red was not used in this experiment. (**B**) Summary data represent 4-α-PDD (5 μM)-induced inward (at −100 mV) and outward (at 100 mV) currents in cECs dialyzed with 0 or 1 mM Mg-ATP. Data represent means ± SEM (*p<*0.05* unpaired Student's t-test vs. the 1 mM Mg-ATP condition, n = 5–9 cECs per condition).
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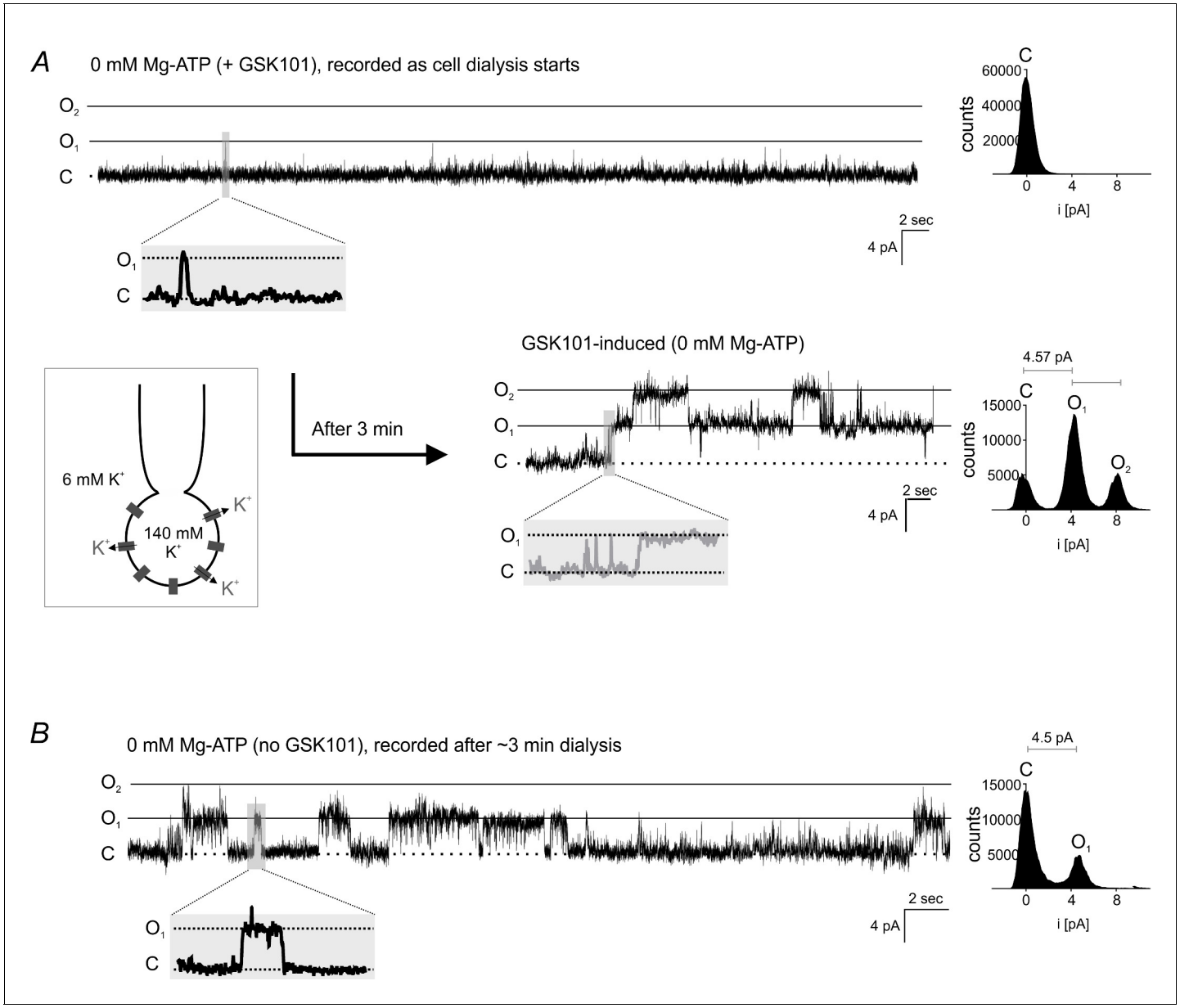

Figure 1—figure supplement 3. Single-channel TRPV4 currents in cECs. (**A**) Agonist-induced single-cell TRPV4 currents in a cEC. *Top*: Representative trace (*left*) and corresponding histogram (*right*) of single-channel activity in a cEC dialyzed with 0 mM Mg-ATP. Currents were recorded in the conventional whole-cell configuration in the presence of 100 nM GSK101 immediately after gaining electrical access to the cell (holding potential, +50 mV; sampling rate, 20 kHz; low-pass filter frequency, 1 kHz). *Bottom*: Trace and histogram for the same cEC recorded ~3 min after initiating dialysis when the effect of GSK101 commenced. The amplitude histogram revealed a unitary TRPV4 current amplitude (i) of 4.57 pA. (**B**) Single-cell TRPV4 currents in the absence of GSK101. Traces and corresponding histogram of single TRPV4 channel currents in a cEC dialyzed with 0 mM Mg-ATP (conventional whole-cell configuration) in the absence of an agonist. The cEC was held at +50 mV, and currents were recorded after dialyzing for ~3 min. The unitary current amplitude was 4.5 pA.

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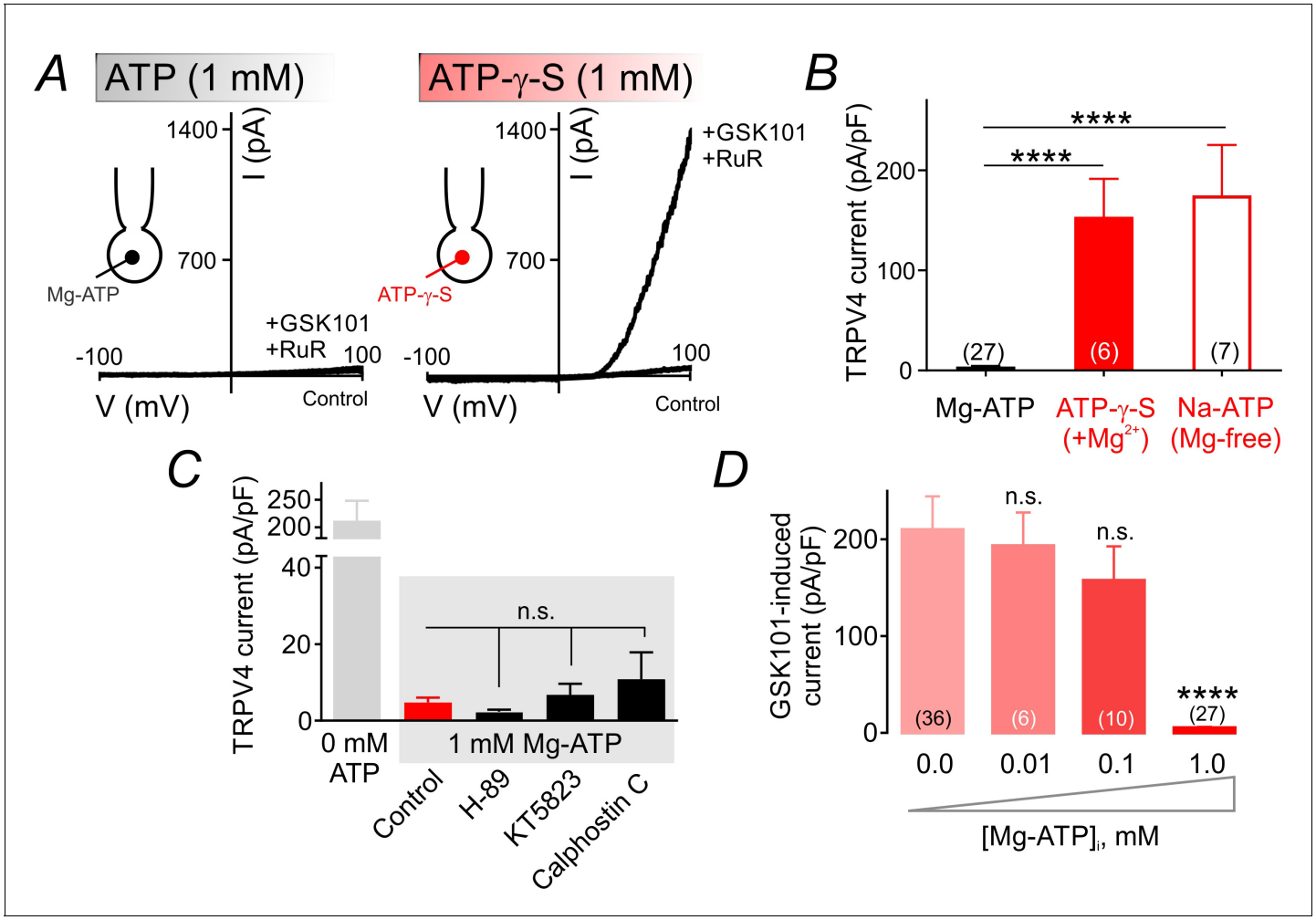

Figure 2. ATP hydrolysis is required for ATP-mediated suppression of TRPV4 channel activity. (**A**) Representative traces of current-voltage relationships in cECs recorded using voltage ramps (−100 to 100 mV) before and after the application of 100 nM GSK101 and 1 μM RuR. cECs were dialyzed with 1 mM Mg-ATP (*left*) or 1 mM Mg-ATP-γ-S (*right*). (**B**) Summary data showing GSK101 (100 nM)-induced outward currents at 100 mV in cECs dialyzed with Mg-ATP (1 mM), Mg-ATP-γ-S (1 mM) or Na-ATP (1 mM, in Mg^{2+} free solution). A minimum duration of 5 min after the application of GSK101 was allowed for outward TRPV4 current to develop in each cEC. Data are presented as means ± SEM (****p<0.0001 vs. Mg-ATP, one-way ANOVA followed by Dunnett's multiple comparisons test). (**C**) TRPV4 outward currents induced by 100 nM GSK101 at 100 mV, recorded from dialyzed cECs (0 and 1 mM Mg-ATP). Mg-ATP–dialyzed cECs (gray shadow) were pre-treated with inhibitors of PKA (H-89, 1 μM), PKG (KT5823, 1 μM) or PKC (calphostin C, 0.5 μM) for ~10–15 min prior to GSK101 application, or left untreated (control). Data are presented as means ± SEM (n.s. denotes not significant vs. control Mg-ATP, one-way ANOVA, Dunnett's multiple comparisons test, n = 6–24). (**D**) Summary data showing the effect of raising intracellular Mg-ATP concentration on GSK101-induced TRPV4 currents. Data are means ± SEM (****p<0.0001, one-way ANOVA followed by Dunnett's multiple comparisons test).

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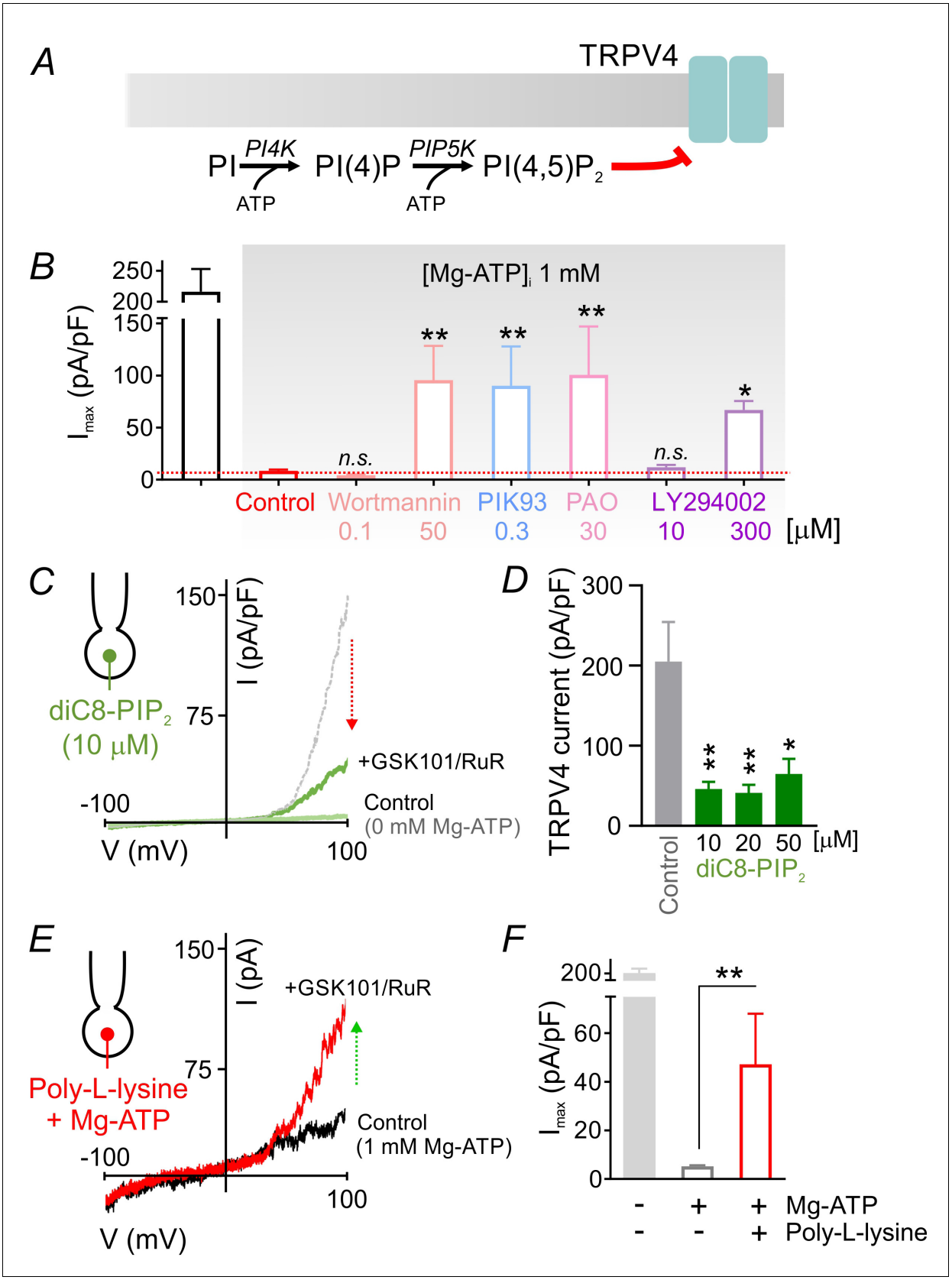

Figure 3. PIP_2 mediates tonic inhibition of capillary TRPV4 channels. (**A**) Schematic diagram showing the ATP-dependent synthesis steps leading to the production of PIP_2. (**B**) Average maximum outward TRPV4 current induced by 100 nM GSK101, recorded in cECs at 100 mV using the conventional

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whole-cell configuration. cECs dialyzed with 1 mM Mg-ATP were treated for ~10 min with wortmannin (0.1, 50 μM), PIK93 (0.3 μM), PAO (30 μM) or LY294002 (10, 300 μM), or were left untreated (control). A minimum duration of 10–15 min after the application of GSK101 was allowed for outward TRPV4 current to develop in each cEC. Data are means ± SEM (**p<0.01, *p<0.05 vs. control Mg-ATP, one-way ANOVA followed by Dunnett's multiple comparisons test; n = 6–27). (**C**) Traces of current-voltage relationship obtained from a cEC dialyzed with 10 μM diC8-PIP_2 and 0 mM Mg-ATP using a voltage ramp (−100 to 100 mV) before and after (green) the application of GSK101 (100 nM) and RuR (1 μM). The dotted gray trace is a representative GSK101-induced current recorded from a control cEC dialyzed with 0 μM diC8-PIP_2 and 0 mM Mg-ATP. (**D**) Summary data showing GSK101 (100 nM)-induced currents at 100 mV in cECs dialyzed with different concentrations of diC8-PIP_2 (10, 20, 50 μM) or 0 μM phosphoinositide (control). The pipette solution lacked Mg-ATP in all groups. GSK101-evoked outward currents developed over ~5 min. Data are presented as means ± SEM (*p<0.05, ***P*<0.01, one-way ANOVA followed by Dunnett's multiple comparisons test; n = 10–18). (**E, F**) Representative trace (**E**) and summary data showing GSK101-induced currents in cECs dialyzed with 1 mM Mg-ATP and poly-L-lysine (3 μg/ml). A duration of 10 min was allowed after the application of GSK101 for outward TRPV4 current to develop in each cEC. Data in F are presented as means ± SEM (**p<0.01, unpaired Student's t-test; n = 8–18).

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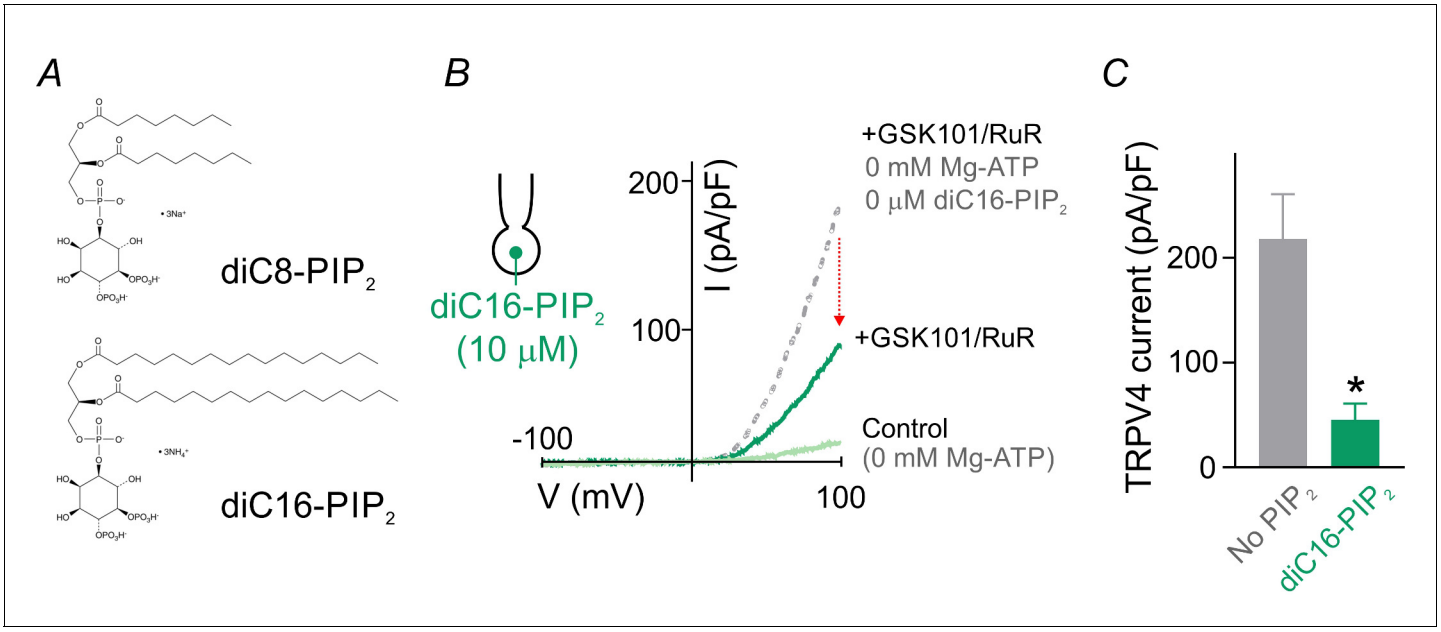

Figure 3—figure supplement 1. The long-acyl chain PIP_2, diC16-PIP_2, suppresses TRPV4 currents. (**A**) Chemical structures of short (dioctanoyl, diC8-PIP_2) and long (dipalmitoyl, diC16-PIP_2) acyl chain PIP_2 salts used in this study. (**B**) Current-voltage relationship represents currents obtained from a cEC dialyzed with 10 μM diC16-PIP_2 and 0 mM Mg-ATP before (light green) and after (dark green) the application of GSK101 (100 nM) and RuR (1 μM), recorded using a voltage ramp (−100 to 100 mV). Outward current developed over 5 min. The dotted gray trace is a representative GSK101-induced current recorded from a control cECs dialyzed with 0 mM diC16-PIP_2 and 0 mM Mg-ATP. (**C**) Summary data showing GSK101 (100 nM)-induced currents at 100 mV in cECs dialyzed with diC16-PIP_2 (10 μM) or 0 μM phosphoinositide (control). The pipette solution lacked Mg-ATP in both groups. Data are presented as means ± SEM (*$p<0.05$, unpaired Student's t-test; n = 3–4 cECs).

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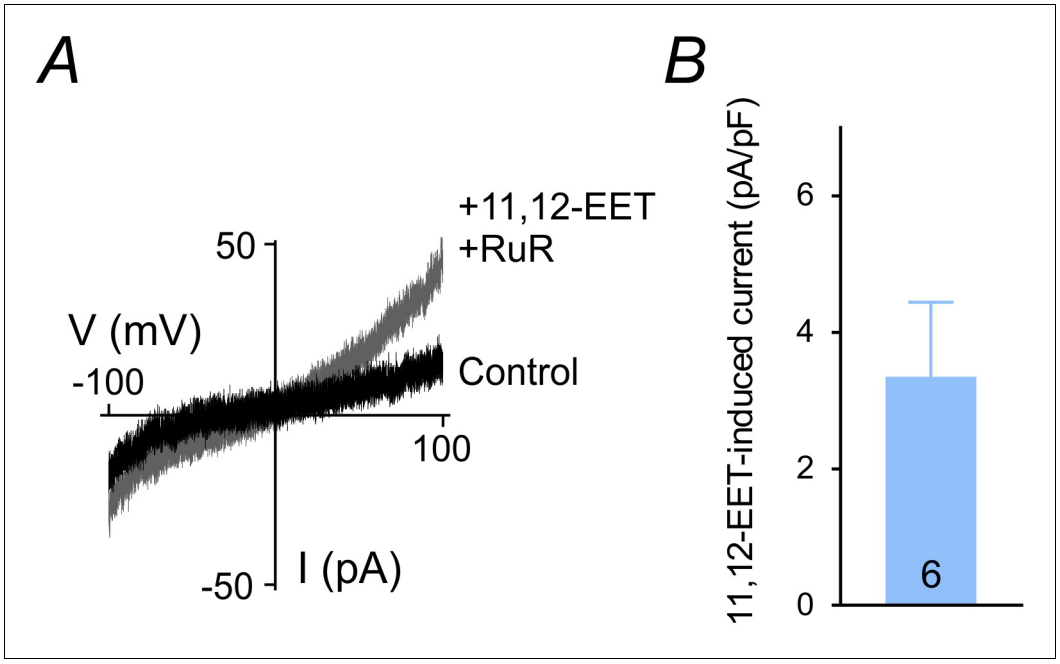

Figure 3—figure supplement 2. 11,12-EET-induced currents in cECs. (**A**) Current-voltage relationship represent currents obtained from a cEC dialyzed with 0 μM diC8-PIP_2 and 0 mM Mg-ATP before (control) and after the application of 11,12-EET (1 μM) and RuR (1 μM), recorded using 300 ms voltage ramps (−100 to 100 mV). (**B**) Summary data showing 11,12-EET-induced outward currents at 100 mV in dialyzed cECs based on experiments in *A* (n = 6 cECs).

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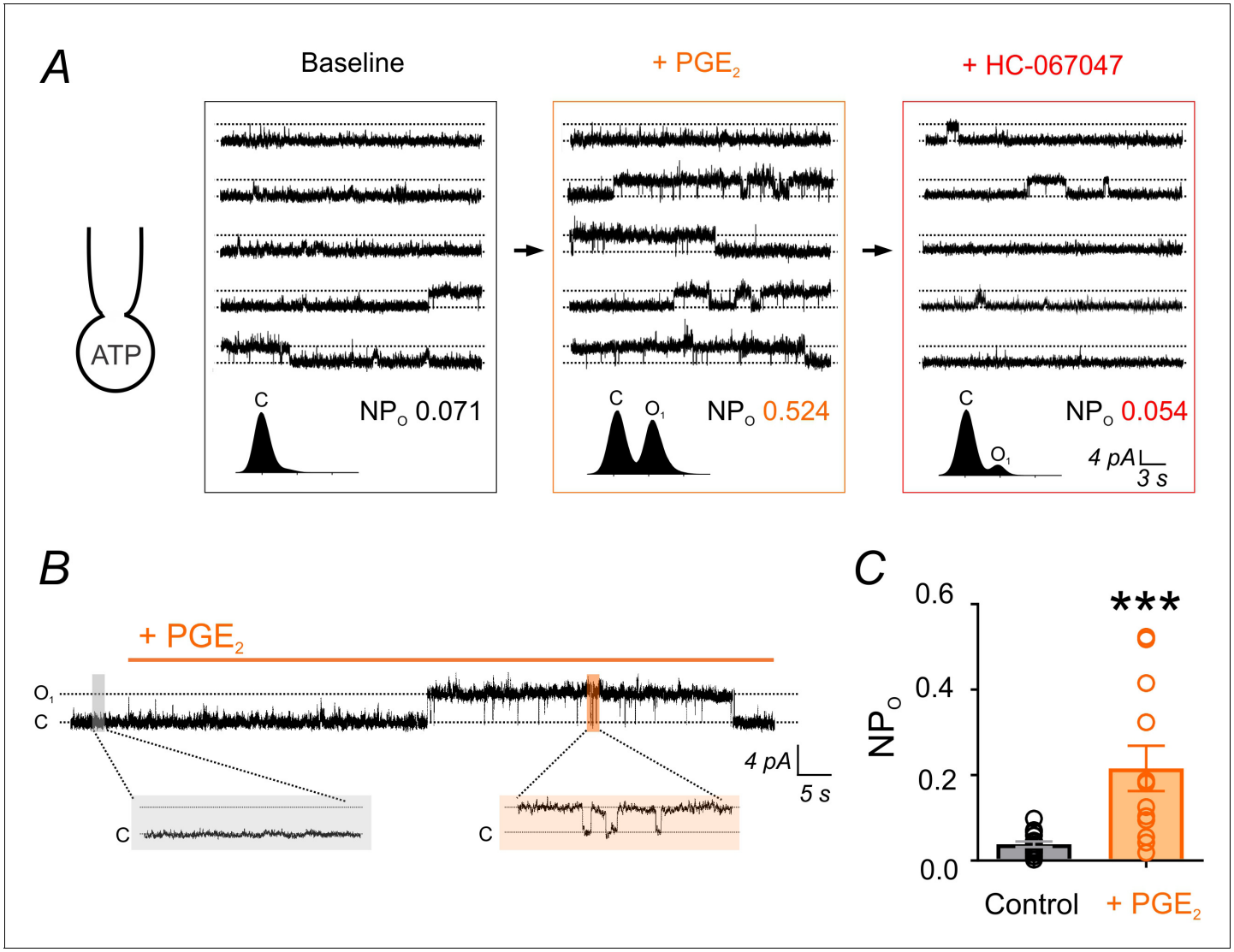

Figure 4. PGE_2 enhances TRPV4 channel activity. (**A**) *Top:* Representative conventional whole-cell recordings from a cEC dialyzed with 1 mM Mg-ATP in the absence of GSK101 and held at a membrane potential of +50 mV. Quantal outward K^+ currents (unitary current, 4.6 pA; sampling rate, 20 kHz; lowpass filter frequency, 1 kHz), reflecting single-channel openings, were recorded before (baseline) and after the consecutive application of PGE_2 (2 µM) and HC-067047 (1 µM). *Bottom:* Corresponding amplitude histograms and open probability (NP_O) values. (**B, C**) Representative trace (**B**) and individual-value plot (**C**) of TRPV4 NP_O in cECs (dialyzed with 1 mM Mg-ATP, held at +50 mV) in the absence (control; n = 16) and presence (n = 12) of 2 µM PGE_2. Data in *C* are means (column bars) ± SEM (error bars, ***p<0.001, unpaired Student's t-test). Each data point represents a recording from a cEC; the average duration of each recording was 5 min.

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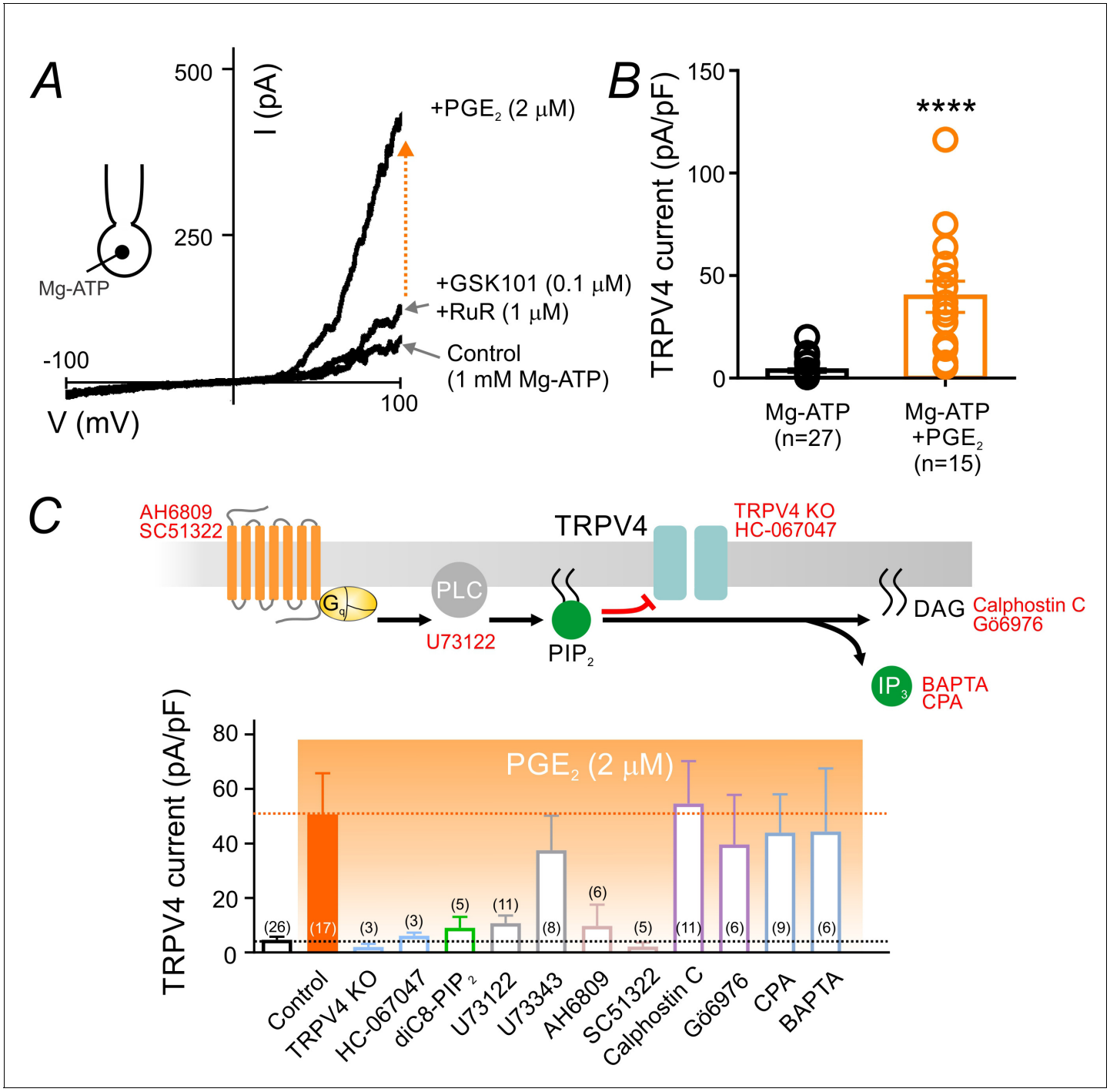

Figure 5. PGE_2 relieves PIP_2-mediated TRPV4 channel suppression. (**A**) Representative current-voltage plots obtained from a cEC dialyzed with 1 mM Mg-ATP and treated consecutively with GSK101 (100 nM) and RuR (1 μM) followed by 2 μM PGE_2. (**B**) Summary individual-value plot of GSK101-induced TRPV4 currents at 100 mV in cECs dialyzed with 1 mM Mg-ATP in the absence (black; n = 27) and presence (orange; n = 15) of 2 μM PGE_2. Incubation of cECs with PGE_2 lasted ~15 min. Data in *B* are means (column bars) ± SEM (error bars, ****p<0.0001, unpaired Student's t-test). (**C**) *Top:* Schematic diagram showing the G_qPCR-dependent hydrolysis of PIP_2 and the interventions used to test different components of the proposed pathway. *Bottom:* Summary data showing GSK101 (100 nM)-induced currents recorded at 100 mV in cECs dialyzed with 1 mM Mg-ATP. Currents were recorded in the absence and presence of 2 μM PGE_2 (orange shading), with or without (control) the indicated interventions. Concentrations (and application method): HC-067047, 1 μM (bath); diC8-PIP_2, 10 μM (pipette), U73122, 10 μM (bath); U73343, 10 μM (bath); AH6809, 10 μM (bath); SC51322, 1 μM (bath); calphostin C, 0.5 μM (bath); Gö6976, 1 μM (bath); CPA, 30 μM (bath); BAPTA, 5.4 mM (pipette). For bath application, pharmacological agents were added 10–15 min before PGE_2 application.

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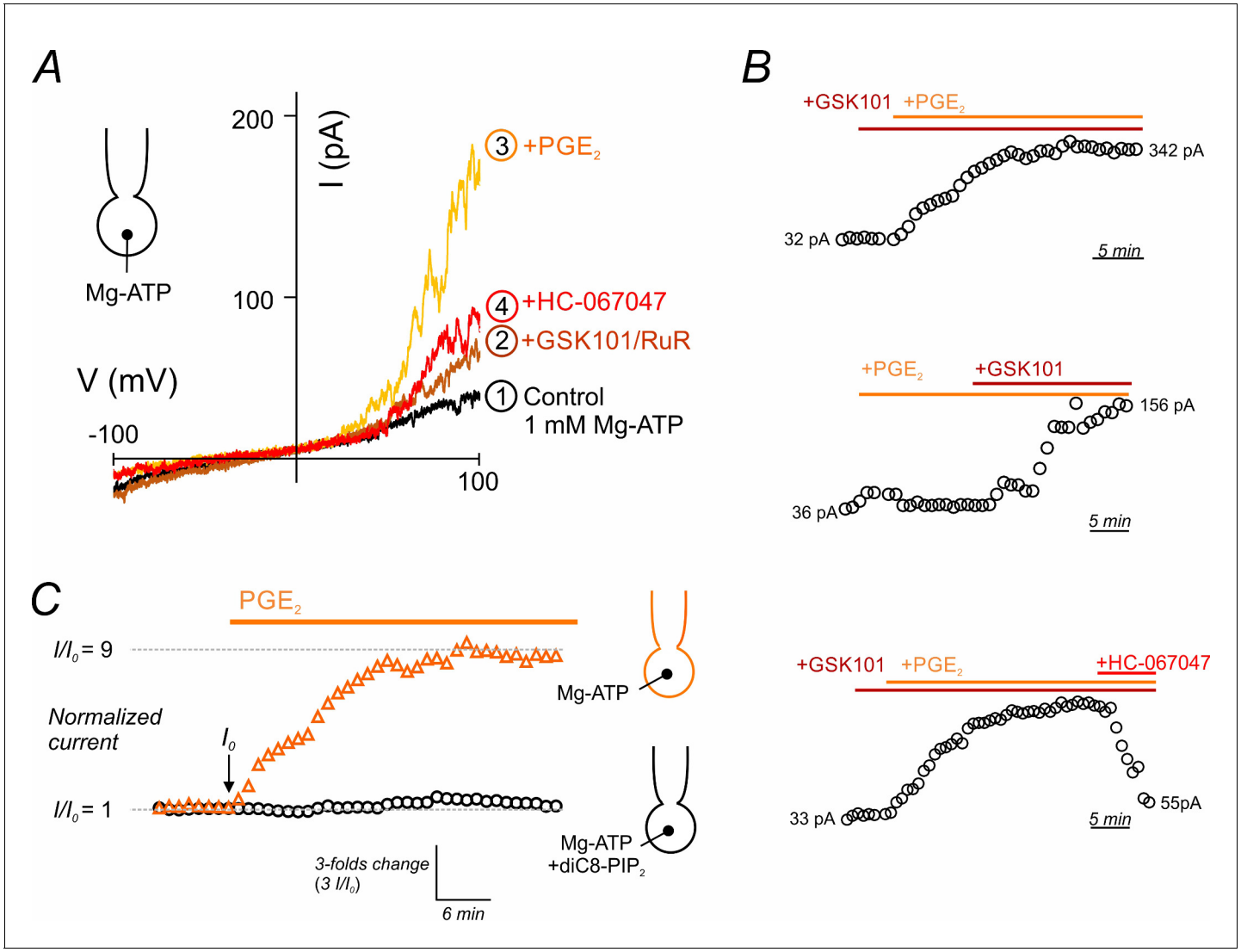

Figure 5—figure supplement 1. PGE_2 relieves TRPV4 current inhibition. (**A**) Current-voltage relationship recorded in a cEC dialyzed with 1 mM Mg-ATP using voltage ramps (−100 to 100 mV). The circled numbers indicated the sequential and cumulative application of GSK101 (100 nM)+RuR (1 μM) followed by 2 μM PGE_2 and lastly, the TRPV4 blocker HC-067047 (1 μM). (**B**) Representative scatter plots of peak current amplitudes (at 100 mV) in three cECs dialyzed with 1 mM Mg-ATP and treated with 100 nM GSK101 followed by 2 μM PGE_2 (or PGE_2 first followed by GSK101). Note that PGE_2-induced activation of TRPV4 current plateaued after ~13–15 min. (**C**) Scatter plots of normalized GSK101-induced outward currents (at 100 mV), normalized to the current at zero time, I_0 (when 2 μM PGE_2 was applied), in two cECs, one dialyzed with 1 mM Mg-ATP (orange symbols) and the other dialyzed with 1 mM Mg-ATP +10 μM diC8-PIP_2 (black symbols). Currents normalized to I_0 are presented before and after the application of PGE_2 for 35 min.

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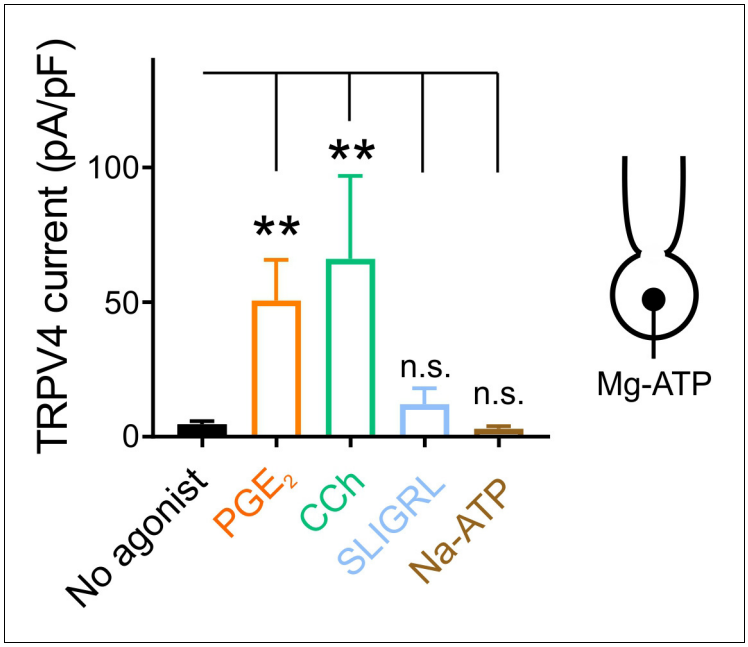

Figure 5—figure supplement 2. Different G_qPCR agonists differentially regulate TRPV4 activity. Summary data showing TRPV4 currents evoked by 100 nM GSK101 at 100 mV in Mg-ATP (1 mM)-dialyzed cECs. Cells were treated with GSK101 alone (no agonist) or together with the G_qPCR agonist PGE_2 (2 μM), carbachol (CCh; 10 μM), SLIGRL (5 μM), or Na-ATP (50 μM). cECs were incubated with different agonists for 15 min while TRPV4 current development was monitored. Data are presented as means ± SEM (**p<0.01, one-way ANOVA followed by Dunnett's multiple comparisons test; n = 11–17 cECs).

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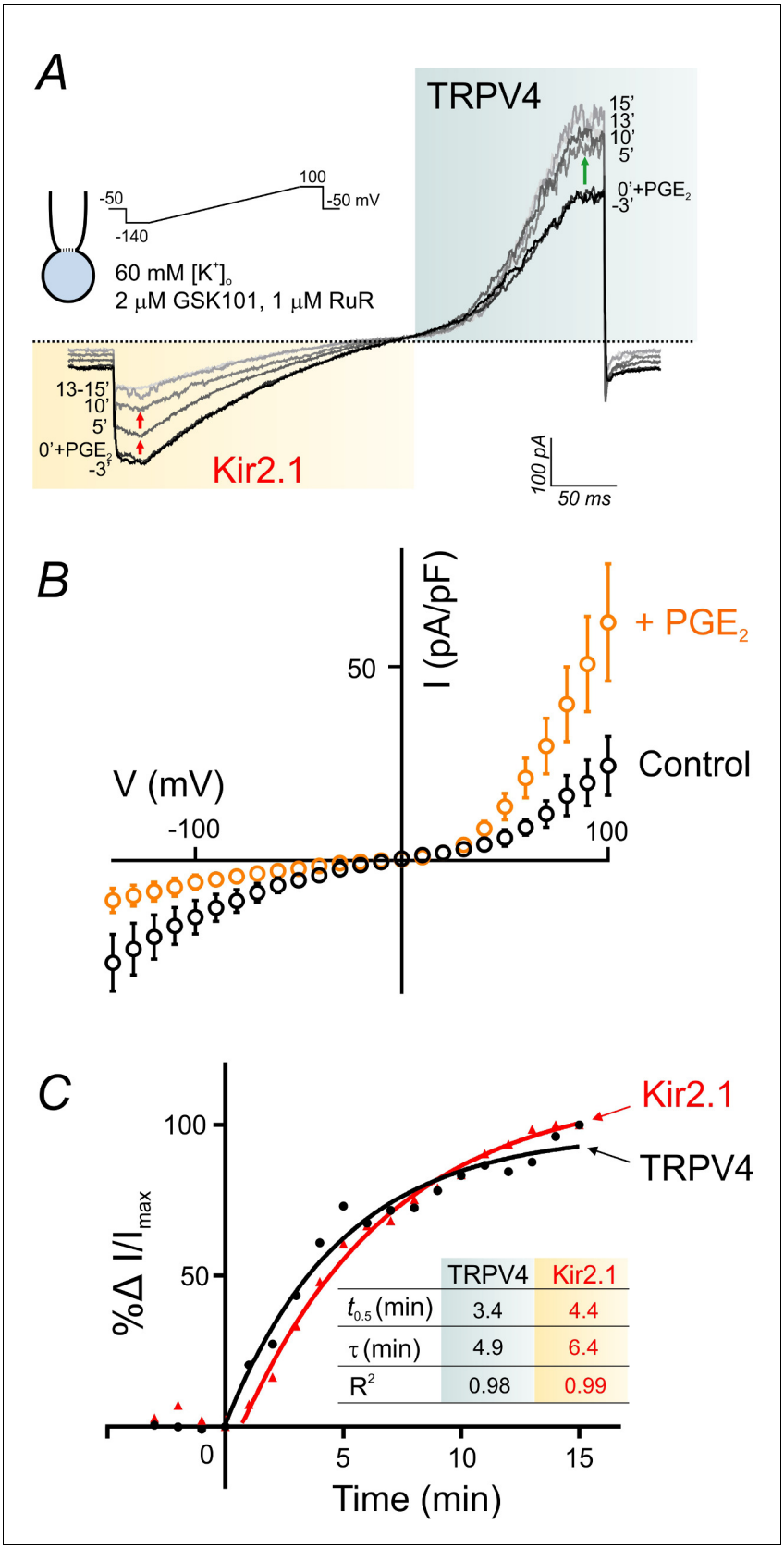

Figure 6. PGE_2 simultaneously and reciprocally regulates TRPV4 and Kir2.1 channel activities. (**A**) Representative traces illustrate simultaneous recordings of Kir2.1 (inward) and TRPV4 (outward) currents in a cEC obtained using the perforated whole-cell configuration. Voltage ramps (300 ms,

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Figure 6 continued

−140 to 100 mV) were used and the cEC was bathed in a 60 mM $[K^+]_o$ solution supplemented with 2 μM GSK101 and 1 μM RuR. Traces represent currents before and for a duration of 15 min after the application of 2 μM PGE_2. (**B**) Averaged current-voltage relationship (n = 5 cECs) corresponding to the experiment in *A*, before (control) and after (maximum changes at 15 min) application of PGE_2. (**C**) Summary data showing the kinetics of TRPV4 current enhancement (black) and Kir2.1 current decline (red) following application of 2 μM PGE_2 onto cECs (as in *A*) at room temperature. Points are average percentage change in normalized currents before and over 15 min after PGE_2 application (n = 5). Curves are best fits of exponential change (rise: TRPV4; decay: Kir2.1). *Inset table:* kinetic parameters based on the two curve fits. Changes in currents plateaued ~13 min after the application of PGE_2.

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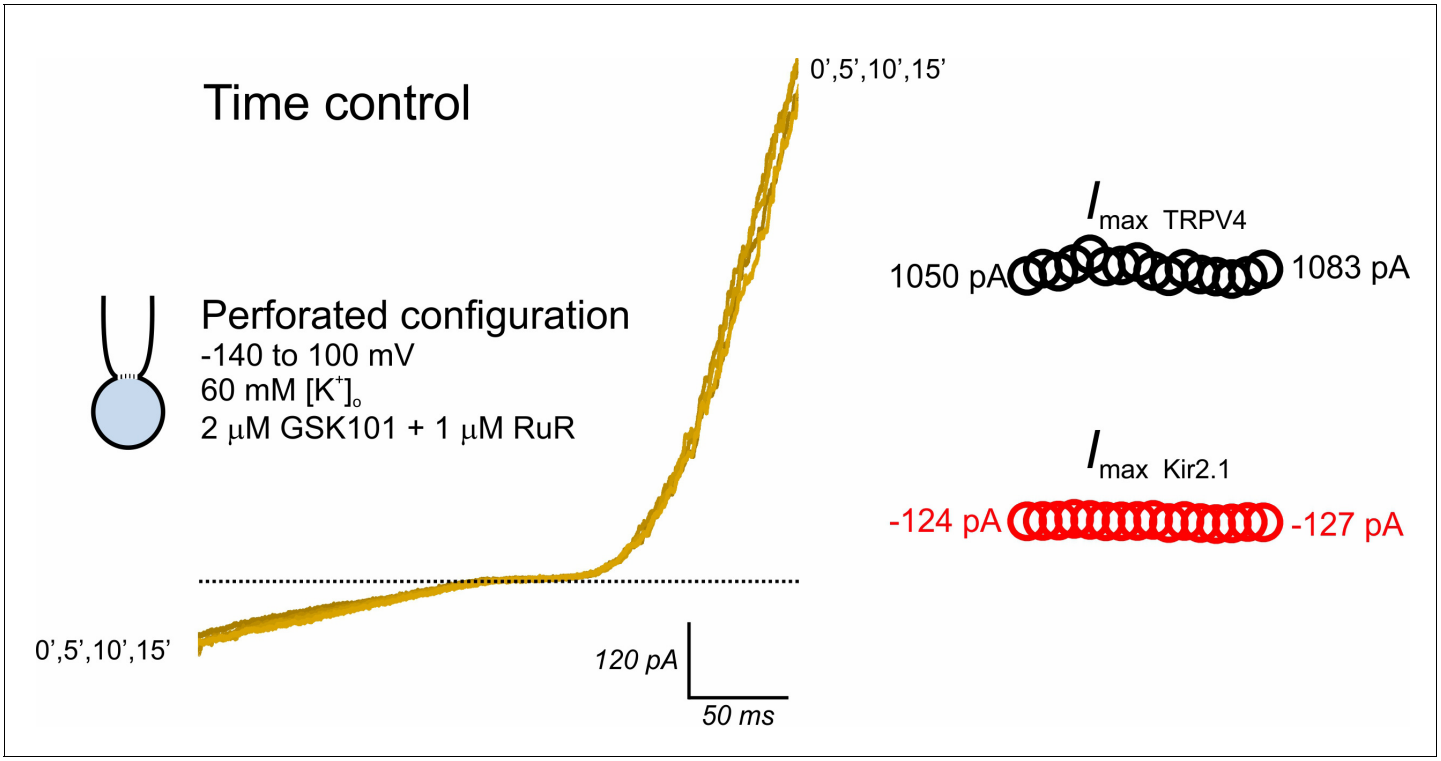

Figure 6—figure supplement 1. TRPV4 and Kir2.1 currents are preserved in cytoplasm-intact cECs. Representative traces obtained from a cEC using the same experimental protocol used in ***Figure 6*** (perforated whole-cell configuration, 60 mM $[K^+]_o$, bath application of 2 μM GSK101 +1 μM RuR). Inward current reflects Kir2.1 activity, and outward current reflects TRPV4 activity. Traces (*left*) and scatter plots (*right*) track inward and outward currents over a duration of 15 min.

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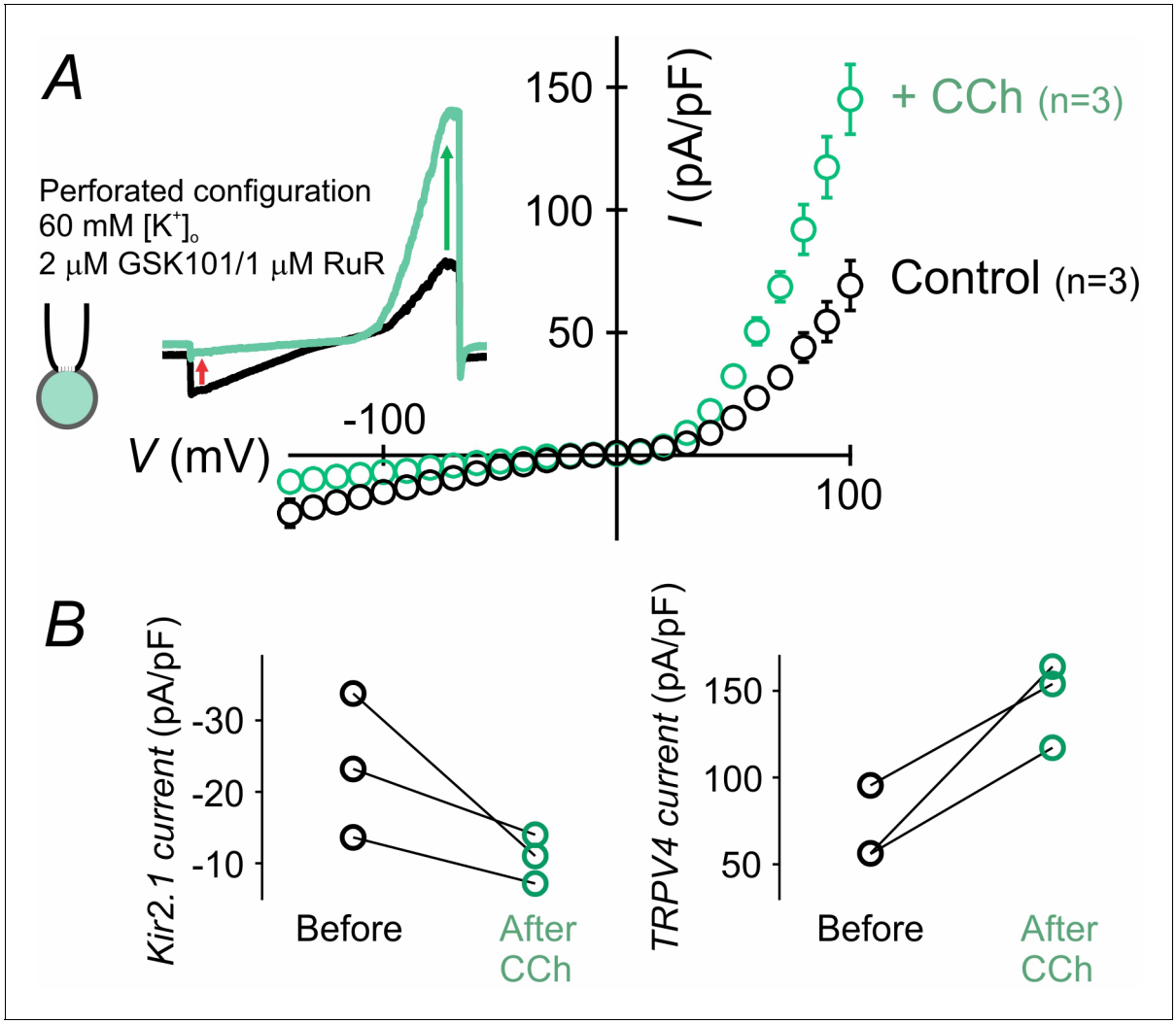

Figure 6—figure supplement 2. The muscarinic receptor agonist carbachol activates TRPV4 currents and inhibits Kir2.1 currents. (**A**) Representative traces and averaged current-voltage relationship for Kir2.1 (inward) and TRPV4 (outward) currents, simultaneously recorded in cECs using the perforated whole-cell configuration. The cEC was bathed in 60 mM $[K^+]_o$ solution supplemented with 2 μM GSK101 and 1 μM RuR, and currents were recorded using voltage ramps (300 ms, −140 to 100 mV). Traces and curves represent current values before and 15 min after the application of carbachol (CCh; 10 μM). (**B**) Paired scatter plots show maximum Kir2.1 (inward at −140 mV) and TRPV4 (outward at 100 mV) current densities before and 15 min after CCh (n = 3 each).

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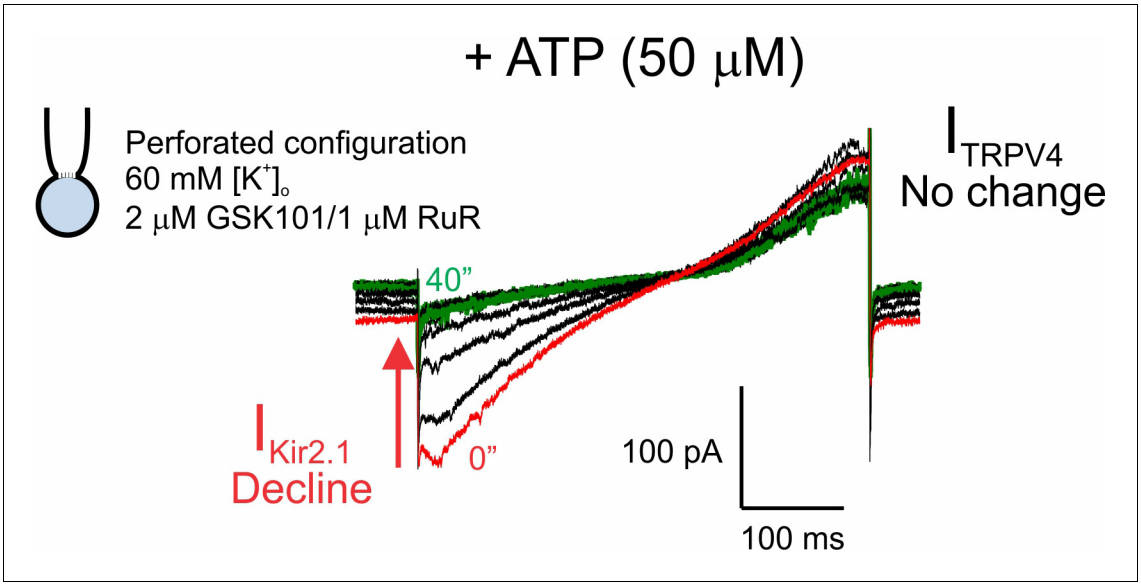

Figure 6—figure supplement 3. Purinergic receptor activation affects Kir2.1 channels but not TRPV4 channels. Representative traces of Kir2.1 (inward) and TRPV4 (outward) currents in a cEC, simultaneously recorded using the perforated whole-cell configuration. The cEC was bathed in 60 mM $[K^+]_o$ solution supplemented with 2 μM GSK101 and 1 μM RuR, and currents were recorded using voltage ramps (300 ms, −140 to 100 mV). Traces represent current values before and at various times up to 40 min after the bath-application of 50 μM Na-ATP.

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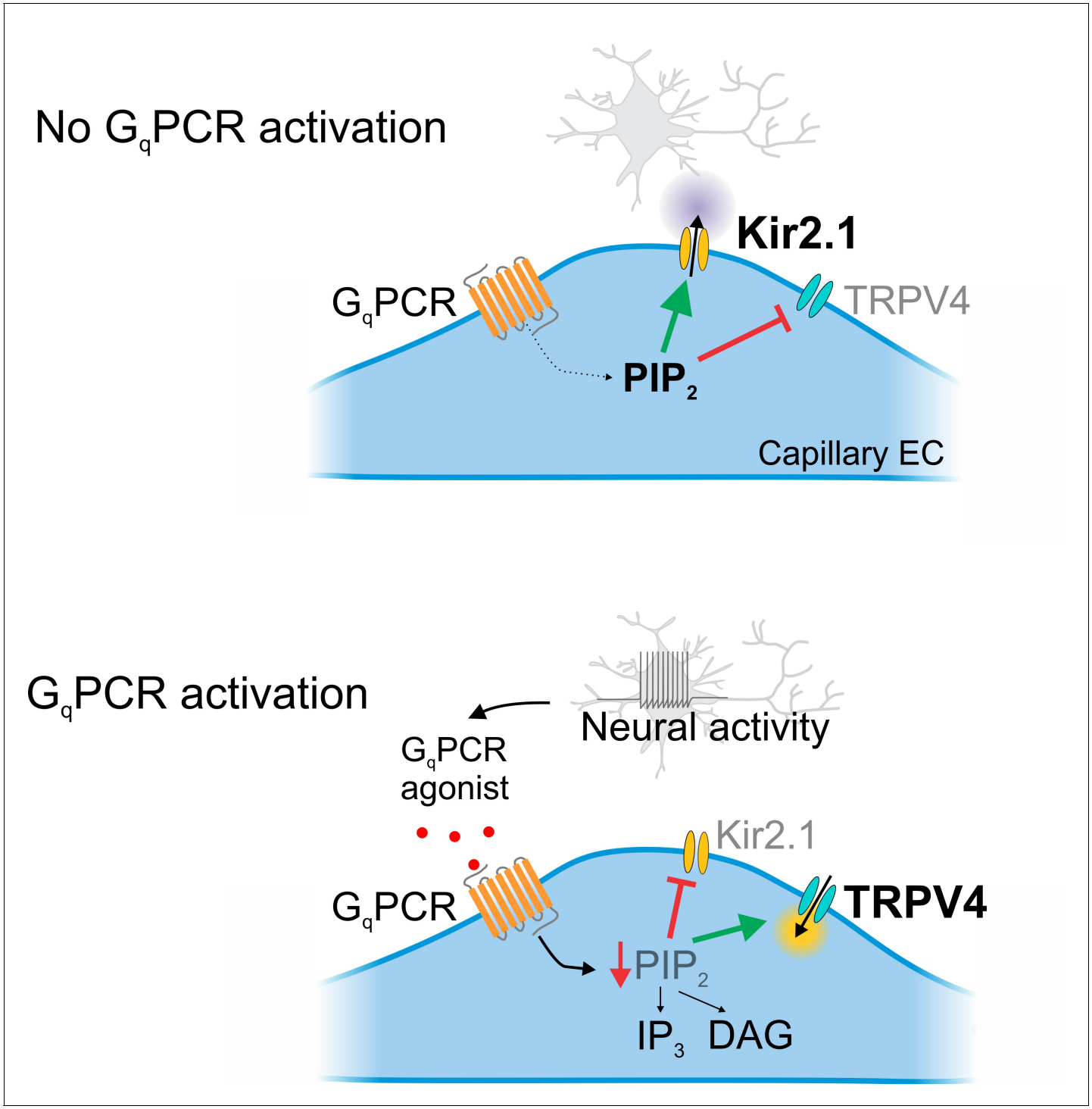

Figure 7. Cartoon representation of G_qPCR-mediated reciprocal effects on capillary ion channel activity. Schematic diagram summarizing the proposed mechanism. *Top*: In the absence of G_qPCR stimulation, endogenous PIP_2 levels are sufficient to tonically inhibit TRPV4 channels and maintain Kir2.1 channel activity. *Bottom*: G_qPCR activation with an agonist stimulates PIP_2 hydrolysis, resulting in the loss of PIP_2-mediated maintenance of Kir2.1 activity and inhibition of TRPV4 activity.

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