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# Synergistic interplay of Gβγ and Phosphatidylinositol 4, 5-bisphosphate dictates Kv7.4 channel activity

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| **Abstract:** | Kv7.4 channels are key determinants of arterial contractility and cochlear mechanosensation that, like all Kv7 channels, have an obligatory requirement for phosphatidylinositol 4, 5-bisphosphate ($PIP_2$). βγ G proteins (Gβγ) have been identified as novel positive regulators of Kv7.4. The present study ascertained whether Gβγ increased Kv7.4 open probability through an increased sensitivity to $PIP_2$. In HEK cells stably expressing Kv7.4 $PIP_2$ or Gβγ increased open probability in a concentration dependent manner. Depleting $PIP_2$ prevented any Gβγ-mediated stimulation whilst an array of Gβγ inhibitors prohibited any $PIP_2$-induced current enhancement. A combination of $PIP_2$ and Gβγ at sub-efficacious concentration increased channel open probability considerably. The stimulatory effects of three Kv7.2-7.5 channel activators was also lost by $PIP_2$ depletion or Gβγ inhibitors. This study alters substantially our understanding of the fundamental processes that dictate Kv7.4 activity, revealing a more complex and subtle paradigm where the reliance on local phosphoinositide is dictated by interaction with Gβγ. |
| **Response to Reviewers:** | The reviewers have been extremely diligent and this has improved our paper considerably. Thank you.<br><br>We have amended all figures as suggested by the Reviewer. The numbers on the expanded inside out traces were actually to denote closed state and multiple open states. This has been added to each legend for clarification.<br><br>All other points have been addressed or corrected. |

**Title: Synergistic interplay of Gβγ and Phosphatidylinositol 4, 5-bisphosphate dictates Kv7.4 channel activity**

**Short title: Gβγ and $PIP_2$ are obligatory for Kv7.4 channel function.**

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**Abstract:**

Kv7.4 channels are key determinants of arterial contractility and cochlear mechanosensation that, like all Kv7 channels, have an obligatory requirement for phosphatidylinositol 4, 5-bisphosphate ($PIP_2$). βγ G proteins (Gβγ) have been identified as novel positive regulators of Kv7.4. The present study ascertained whether Gβγ increased Kv7.4 open probability through an increased sensitivity to $PIP_2$. In HEK cells stably expressing Kv7.4 $PIP_2$ or Gβγ increased open probability in a concentration dependent manner. Depleting $PIP_2$ prevented any Gβγ-mediated stimulation whilst an array of Gβγ inhibitors prohibited any $PIP_2$-induced current enhancement. A combination of $PIP_2$ and Gβγ at sub-efficacious concentration increased channel open probability considerably. The stimulatory effects of three Kv7.2-7.5 channel activators was also lost by $PIP_2$ depletion or Gβγ inhibitors. This study alters substantially our understanding of the fundamental processes that dictate Kv7.4 activity, revealing a more complex and subtle paradigm where the reliance on local phosphoinositide is dictated by interaction with Gβγ.

**Keywords:** Potassium channel, KCNQ, $PIP_2$, G-protein βγ, ion channel regulation

**Introduction:**

The Kv7 family of potassium channels (Kv7.1-Kv7.5) are crucial determinants of cardiac, neuronal, cochlear and vascular function [25, 26]. As such, defining the mechanisms that control how Kv7 channel activity is regulated is crucial. It is acknowledged that Kv7 channels have an obligatory requirement for phosphatidylinositol 4, 5-bisphosphate ($PIP_2$) [4, 12, 18] but we recently described how the Kv7.4 channel, important for normal vascular function [6, 7, 15], requires G-protein βγ subunits (Gβγ) for its voltage dependent

activity [27]. Few ion channels are directly regulated by Gβγ with the best studied being the G protein coupled inwardly rectifying potassium (GIRK) channel that controls electrical excitability in neurons and cardiac cells [9-11, 19]. This channel is comprised of Kir3.1 - 3.4 subunits and Gβγ enhance open probability by stabilizing the interaction of $PIP_2$ [9, 13, 20]. We speculated whether the stimulatory effects of Gβγ on Kv7.4 was due to an interaction with $PIP_2$ analogous to GIRK channels. As such, Gβγ would be ineffective under conditions where $PIP_2$ levels were reduced. However, the study revealed a level of regulation far more complex and provides the first account of a synergistic regulation of Kv7.4 channels by Gβγ and $PIP_2$.

**Methods:**

*Cell culture.* Human embryonic kidney cells (HEK293) stably transfected with Kv7.4 were maintained in modified Eagles' medium containing 10% Foetal calf serum, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% L-glutamine and 1% sodium pyruvate. For experiments, cells were detached by brief trypsin treatment. HEK Kv7.4 cells were plated on 13mm coverslips in an external physiological salt solution (PSS) containing (mM): KCl 6, NaCl 120, $MgCl_2$ 1.2, $CaCl_2$ 2.5, D-glucose 12 and HEPES 10, pH was adjusted to 7.35 with NaOH. Cells were left on cover slips for 30mins at room temperature and stored at $4^{O}C$ for up to 8 hours.

*Whole-cell Electrophysiology.* Macroscopic transmembrane ionic currents of HEK293 Kv7.4 cells were recorded using standard amphotericin B (300 μg/ml) perforated-patch techniques in voltage-clamp mode. In some experiments the ruptured whole-cell patch-clamp technique was used for intracellular perfusion of active Gβγ subunits. Patch

pipettes were fire-polished and had a resistance of 4-8 MΩ when filled with the pipette solution of the following composition (mM): KCl 126, $MgCl_2$ 1.2, HEPES 10, EGTA 0.5, pH was adjusted to 7.2 with KOH. 1 mM of $Na_2ATP$ was added to the pipette solution for the ruptured whole-cell experiments. Cells were held at -60 mV and current amplitude was monitored by application of test pulse to +20 mV. To generate current-voltage relationships a voltage step protocol was used from a holding potential of -60 mV testing a range of voltages from -90 to +40 mV in 10mv increments at 15s intervals. Drugs were applied in the external solution using a bath perfusion system, except for Gβγ subunits which were included in the pipette solution.

*Single channel electrophysiology*. Single channel activity of Kv7.4 currents expressed in HEK293 cells was recorded in voltage-clamp mode using inside-out patch configuration in an external solution of the following composition (mM): KCl 165, HEPES 5, EGTA 10, pH was adjusted to 7.2 with NaOH. Patch pipettes were fire-polished and had a resistance of around 20 MΩ when filled with PSS as a pipette solution. Cells were voltage clamped at 0mV. Cell-attached patch configuration was used in some experiments. In this case PSS was used for both, pipette and bath solutions and the cells were recorded at -50mV, so the expected membrane potential under the patch is around -6mV (assuming resting membrane potential of -56mV [21]). All single-channel current records were filtered at 0.1 kHz using a Frequency Devices 9002 digital filter with 8-pole lowpass Bessel filter and acquired at 1 kHz (Axopatch 200B 4-pole lowpass Bessel filter). Current amplitudes were calculated from idealized traces of ≥ 180s in duration using 50% threshold method using pClamp 9.0 software. Events lasting less than 6.664 ms (2 × rise time for a 100 Hz, -3 db, low pass filter, [8]) were excluded from the analysis to maximize

the number of channel openings reaching their full current amplitude. Channel activity was expressed as NPo, which was calculated automatically and reported by the pClamp 9.0 software under "event statistics" after completion of the single-channel search procedure applied to the idealized traces ≥ 180s in duration at each condition. All whole-cell and single channel current recordings were made using AXOpatch 200B amplifier (Axon Instruments) at room temperature. Electrical signals were generated and digitized using a Digidata 1322A hosted by a PC running pClamp 9.0 software (Molecular Devices). Drugs were applied in the external solution using a push-pull system. All electrophysiological data were analysed and plotted using pClamp 9.0, MicroCal Origin 6.0 (MicroCal Software, Northampton, MA, USA) and GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). $PIP_2$ and βγ G proteins were applied at a range of concentrations to inside-out excised patches to determine the sensitivity of the Kv7.4 channel to these modulators. However, it was not usually possible to hold the patch long enough to do a full concentration-response and saturating responses were not achieved. Data are therefore accumulated from a number of patches and plotted by a sigmoidal curve to give an approximate estimate of sensitivity. Values for half maximal stimulation taken from these fits are quoted in the text with the caveat that saturation of channel enhancement was not seen with either molecule.

*In-cell Western blot.* In-cell Western blot experiments were performed as described elsewhere [5]. HEK293 cells stably expressing Kv7.4 were grown in 96-well plates for 24h and incubated with different drugs as indicated. After treatment, cells were fixed with 3% ice-cold paraformaldehyde for 10 min at room temperature (RT, 22-24°C), washed with PBS and blocked/permeablised with PBS containing 5% bovine serum albumin

(BSA) and 0.2% Triton X-100 for 1h at RT. Cells were subsequently incubated for 14-16h at 4°C with the following primary antibodies: mouse anti $PIP_2$ (2C11, dil 1:200, Santa Cruz, Dallas, USA) and rabbit anti cytochrome c oxidase subunit IV (COX-IV, dil 1:1000, Abcam, Cambridge, UK). After 3 washes with PBS (10min each), cells were incubated with anti-mouse and anti-rabbit IgG conjugated to IRDye® 680RD and IRDye® 800CW, respectively (dil 1:1000, Li-Cor, Cambridge, UK), for 1h at RT. All antibodies were diluted in PBS containing 1% BSA and 0.04% Triton X-100. Cells were then washed 3 times with PBS and imaged on the Odyssey Infrared Imaging System (Li-Cor, Cambridge, UK) and analysed with supplier's software (Version 3.1).

*Statistical analysis.* All data are mean ± s.e.m. of n cells. One-way ANOVA test followed by a Dunnett's or Tukey's multiple comparisons test or Student's t-test were used to determine statistical significance between groups, where * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ and **** = $P < 0.0001$.

***Reagents.*** Many different pharmacological tools were used to either alter $PIP_2$ levels or impair βγ G protein interactions. These are listed below with the supplier and mechanism of action.

*$PIP_2$ depletion*. Wortmannin (Sigma Aldrich, Poole, UK), at 20 µM is an inhibitor of myosin light chain kinase, phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase. It depletes $PIP_2$ levels by inhibiting synthesis from phosphatidyl inositol via the phosphatidylinositol 4-kinase [1, 21, 28]. $PIP_2$ depletion was augmented by brief application of 100 µM trypsin that activates protease activated receptors endogenous to the HEK cell [30]. In addition, the phospholipid acceptor α-Cyclodextrin, and LY-

294,002 hydrochloride, another inhibitor of phosphatidylinositol kinase (both from Sigma) were used for in-cell western blot studies.

*Prevention of βγ G protein interaction.* We used a range of structurally different compounds that prevent βγ G proteins interacting with effector proteins through different mechanisms. Gβγ-target recognition is defined by a single "hotspot", which has distinct sub-surfaces for individual G protein subunit interactions [3]. Gallein (Tocris, Avonmouth, UK), M201 and M119K (provided by National Cancer Institute Drug Development Programme, 3) all bind to the hot spot at concentrations less than 1 μM but differentially modulate Gβγ interactions with effectors [3]. Grk2i (Tocris, Avonmouth, UK, 10 μM) is a peptide analogue of the G-protein receptor kinase c-terminus [16] which competes with effector proteins for βγ G protein binding. All reagents were applied to the bathing solution at concentrations derived from previous publications.

*Additional Materials.* G-protein βγ subunits from bovine brain was purchased from Merck. $PIP_2$ (D-*myo*-Phosphatidylinositol 4,5-bisphosphate) was purchased from Echelon. HEK293 cells stably expressing Kv7.4 were a gift from the University of Copenhagen [2, 24]. Retigabine, S-1 and NS15370 were synthesised by NeuroSearch A/S, (Ballerup, Denmark). The pan-Kv7 blocker linopirdine was purchased from Tocris (Avonmouth, UK).

**Results:**

*$PIP_2$ depletion abolishes Kv7.4 currents and prevents their activation by Gβγ.*

In HEK cells stably expressing Kv7.4, depolarisation evoked characteristic time-dependent currents (Fig 1) that were abolished by treatment with the Kv7 blocker

linopirdine (10 μM) and were not apparent in untransfected HEK cells [27]. Application of 20 μM wortmannin, to reduce $PIP_2$ levels gradually decreased Kv7.4 currents recorded in perforated-patch whole cell configuration (Fig. 1A) Further inhibition of currents to a level identical to that recorded after application of the Kv7 channel blocker linopirdine (10 μM) was achieved by brief (≤ 30 s) application of 1 μM trypsin to stimulate endogenous G-protein-coupled proteinase-activated receptors (Fig 1B). Wortmannin also reduced Kv7.4 channel activity in cell-attached experiments (Fig 1C). In-cell Western analysis showed that wortmannin alone and in the combination with trypsin application significantly decreased levels of $PIP_2$ in Kv7.4 transfected HEK cells (Fig 1D). We then investigated whether $PIP_2$ depletion modified the stimulatory response to enrichment of internal solutions with Gβγ. Like our previous study [27] intracellular perfusion of active Gβγ (250 ng/ml) increased current amplitude by ~70 % within 5 mins of rupture in control cells (Fig 2Aa, B) but had considerably less effect (~38 % increase) in cells incubated with 20 μM wortmannin (Fig 2Ab, B). We then undertook inside-out excised patch recording to investigate this effect further. In HEK cells stably expressing Kv7.4 but not untransfected cells robust $K^+$ channel activity was recorded immediately upon patch excision that usually decayed within 1-2 minutes to a steady, lower level of activity. This is considered to be due to the wash out of key intracellular mediators from the membrane patch [4, 12, 18]. Bath application of Gβγ (0.4-50 ng/ml) increased channel activity in a concentration-dependent manner similar to previous work [27] with an estimated value for half maximal stimulation of 8.1 ng/ml (n=4-7, Fig. 3A, B). However, bath application of Gβγ had no effect (2.51 ± 0.05% increase only, n=4) on the negligible channel activity recorded in the continued presence of wortmannin (Fig 3C) whereas application of exogenous $PIP_2$ in the continued presence of wortmannin re-established channel activity

(Fig. 3D, n=5). These data show that inhibition of $PIP_2$ re-synthesis by wortmannin decreased $PIP_2$, reduced channel activity and prevented Gβγ –mediated channel stimulation suggesting that Gβγ may act upstream of $PIP_2$.

*Gβγ inhibition abolishes Kv7.4 currents and prevents their activation by $PIP_2$.*

Having established that $PIP_2$ depletion impaired Gβγ-mediated enhancement of Kv7.4 currents we addressed whether reduction of Gβγ activity limited the well-established $PIP_2$-enhancement of channels. Initially we used the in-cell Western blot technique to ascertain whether Gβγ inhibitors altered $PIP_2$ levels. Figure 4A shows that neither Grk2i, M201 nor M199K altered $PIP_2$ levels whilst the well-used small molecule Gβγ inhibitor gallein reduced $PIP_2$ to some extent. In a previous study, we showed that gallein, Grk2i and an antibody raised against Gβ inhibited whole cell Kv7.4 currents markedly [27]. Application of two novel and potent Gβγ inhibitors M201 and M119K [3] also inhibited whole cell Kv7.4 currents significantly (Fig. 4B, C). Figure 4C shows the accumulated data for all Gβγ inhibitors on whole cell Kv7.4 currents highlighting the suppressive effect irrespective of mechanistic action. Having established the effect of Gβγ inhibitors on whole cell currents we then performed inside-out recordings to determine how single channel activity was affected. Figure 4 D and E shows that M201 and Grk2i reduced the open probability of Kv7.4 channels in excised patches to negligible levels similarly to the action of gallein [27] (Fig. 4F).

Having confirmed that inhibition of Gβγ inhibited Kv7.4 currents recorded at the whole cell and single channel level we used inside-out excised patches to ascertain if structurally different Gβγ inhibitors altered $PIP_2$-induced enhancement of Kv7.4 activity. Under control conditions application of $PIP_2$ increased the apparent channel open probability (NPo) of the Kv7.4 channel activity in a concentration-dependent manner (Fig

5A, B). This resulted in an estimated value for half-maximal stimulation of 117 μM (n=4-11) similar to previous papers [4, 12, 18]. By comparison, no significant increase in Kv7.4 channel activity was observed upon application of $PIP_2$ (10-100 μM) in patches incubated in M201, gallein or Grk2i (Fig 5C-F). These data reveal that inhibition of Gβγ interaction by structurally and mechanistically different agents not only inhibits Kv-7.4 basal activity, but also prevented $PIP_2$ stimulation of Kv7.4 (Fig 5F).

*$PIP_2$ and Gβγ are synergistic regulators of Kv7.4 channels.*

Our data thus far suggest that rather than regulation of Kv7.4 by $PIP_2$ and Gβγ being a linear relationship the signal molecules combine to dictate channel function. The next series of inside-out experiments aimed to determine if a low sub-effective concentration of Gβγ could enhance the sensitivity of the channel to exogenous $PIP_2$. Application of low concentrations of either Gβγ (1 ng/ml) or $PIP_2$ (1-3 μM) to inside-out patches had a negligible effect on Kv7.4 channel activity (Fig 6). However, Figure 6 B-D shows that in combination a marked increase in channel activity was observed and the presence of 1 ng/ml Gβγ increased the sensitivity of the channel to $PIP_2$. For instance, NPo increased to 0.29 ± 0.07 (n=6) when 3 μM $PIP_2$ was applied in the presence of 1 ng/ml Gβγ, which was significantly greater than NPo when 3 μM of $PIP_2$ alone was applied (0.077 ± 0.02, n=8, Fig 6C). The stimulatory effect of Gβγ was not apparent when 100 μM $PIP_2$ was applied suggesting the combinational effect had a functional ceiling (Fig 6D). Consequently, low concentrations of Gβγ produced a leftward shift in the sensitivity of the Kv7.4 channel to $PIP_2$ suggesting the two molecules acted synergistically.

*$PIP_2$ depletion and reduced Gβγ activity affects pharmacological modulation of Kv7.4.*

Application of three structurally different enhancers of Kv7.2-7.5, S-1, retigabine and NS15370, produced a marked increase in whole cell currents in HEK cells stably expressing Kv7.4 with currents at +20 mV increasing by approximately 40 % (Fig 7). In cells treated with wortmannin alone some enhancement with each activator was observed. However, in cells bathed in either wortmannin plus trypsin or gallein alone the stimulatory effect of all three agents was abrogated. Consequently, the ability of pharmacological agents to augment Kv7.4 was compromised by $PIP_2$ depletion or reduced βγ subunit activity.

**Discussion:**

It is accepted dogma that Kv7 channels have an obligatory reliance on $PIP_2$ for effective function. We now reveal that this positive regulation occurs synergistically with Gβγ and there is an obligatory reliance on both mediators to be present for effective channel function. These findings change radically our understanding on how Kv7.4 channels are regulated. If other Kv7 channels are also affected by Gβγ in a similar manner the findings of the present study will have wide ranging implications as Kv7 channels regulate cellular physiology in many cell types. We show by in-cell Western blot that treatment with the phosphatidylinositol-4-kinase inhibitor wortmannin and stimulation of G-protein coupled receptors with trypsin reduced global $PIP_2$, and reduced Kv7.4 currents to negligible levels (Fig 1). More importantly this treatment abrogated the stimulatory effect of Gβγ on Kv7.4 channels but did not impair the ability of exogenous 100 μM $PIP_2$ (Fig 3) to enhance Kv7.4 channel activity in excised patches. This implied $PIP_2$ sensitization underlies the positive effect of Gβγ. However, treatment with structurally and mechanistically different inhibitors of Gβγ interactions (gallein, M201, M199K and Grk2i) also decreased Kv7.4 channel activity (Figs 3 & 4) to the same levels as treatment

with wortmannin and trypsin, which mirrored the inhibition produced by the Kv7 channel blocker linopirdine. Moreover, all Gβγ inhibitors prevented any increase in channel activity by exogenous $PIP_2$ (100 μM) in excised patches (Fig 5). It is worth stressing that these inhibitors disable Gβγ interactions with target proteins through different binding domains and mechanisms [3] consistent with Gβγ having differential protein effector sites but all suppressed $PIP_2$-induced enhancement of Kv7.4 open probability. These findings suggested that $PIP_2$ was not simply an upstream channel regulator. In fact, these findings reveal that inherent Kv7.4 channel activity is dictated by a coordinated interaction with $PIP_2$ and Gβγ. Removal of either regulatory arm leads to progressive run down of channel activity and refractoriness to stimulation by either molecule. The synergistic effect of $PIP_2$ and Gβγ was substantiated by the observation that a sub-efficacious dose of Gβγ (1 ng/ml) potentiated the response to a low concentration of $PIP_2$ (1-10 μM) but not a saturating dose of $PIP_2$ (100 μM) resulting in a pronounced leftward shirt in the response to exogenous $PIP_2$ (Fig 6).

In addition to the control of Kv7.4 activity at rest we also show that compounds that activate Kv7.2-7.5 through a common molecular mechanism [14, 17, 22, 33] also required $PIP_2$ and Gβγ binding to be effective. These findings corroborate previous work that showed retigabine-induced stimulation of Kv7.2/ or 7.3, which comprise the neuronal M-channel, was negligible after $PIP_2$ depletion [35]. Interestingly the pharmacological dependence on $PIP_2$ was localized to an interaction site more proximal than the well-established C-terminus site necessary for channel activity [35]. It is possible that the synergistic effect of Gβγ is due to alterations of endogenous levels of $PIP_2$ and Gβγ do activate phospholipase Cβ and Phosphoinositide-3-kinase [23]. However, these enzymes would reduce rather than enhance $PIP_2$ levels. Moreover, the Gβγ inhibitors M201,

M199K and Grk2i had no discernible effect on $PIP_2$ levels whereas gallein decreased global $PIP_2$. This effect of gallein has not been reported previously and there is no reason for the effect. The fact that other Gβγ inhibitors did not alter global $PIP_2$ levels suggests it is a quirk of the molecule rather than of Gβγ inhibition.

The dual regulation of Kv7.4 by $PIP_2$ and Gβγ identified in the present study bears a considerable similarity to GIRK channels, which have a co-dependence on Gβγ and $PIP_2$ [10, 20]. Early studies proposed that Gβγ stabilized the $PIP_2$ interaction with Kir3.1 / Kir3.4 [13, 29]. Crystallographic studies revealed that full GIRK activation was reliant upon $PIP_2$ interacting with an internal gate independent of the G-gate where Gβγ bind and no channel openings occurred if either gate was unoccupied [31, 32]. Currently the same level of molecular insight does not exist for Kv7.4. $PIP_2$ binding domains have been identified in other Kv7 family members but this information is lacking for Kv7.4. In addition, there is no information on binding sites for Gβγ. In GIRK channels approximately 12 sites across the protein have been identified as important for Gβγ binding that combine to accommodate 4 Gβγ molecules in the functional tetramer [9, 34]. Moreover, distinct high- and low-affinity sites exist in GIRKs that determine basal activation and receptor-mediated activation, respectively [11]. These aspects of molecular recognition need to be determined for Kv7.4.

A criticism that could be levelled at this study is it relies solely on pharmacological agents for the conclusion. However, we use blockers that are not only structurally different but which work through varied mechanisms. As wortmannin and various inhibitors of Gβγ reduced Kv7.4 channel activity to negligible levels there must be sufficient $PIP_2$ and Gβγ in the channel locality to sustain channel activity under excised patch conditions. It is generally assumed that $PIP_2$ levels remain consistent in a normal

healthy cell and are replenished rapidly upon hydrolysis. Our data show that Kv7.4 activity is attenuated by Gβγ inhibitors implying that there is a persistent influence of Gβγ maintaining channel activity. Again, there is a parallel with GIRK channels that have a basal level of activity due to constitutive binding of Gβγ [11]. The free Gβγ maintaining Kv7.4 activity may be the product of binding of Gα to the channel protein and the associated tethering of Gβγ as shown for Kir3.1 [11], localised G-protein coupled receptor activity or a labile free pool of Gβγ. Irrespective of these unknowns we have identified that Kv7.4 activity is crucially dependent on a synergistic interplay between $PIP_2$ and Gβγ. Kv7 channels are well known to have an obligatory requirement for $PIP_2$ but this study reveals a more complex and subtle paradigm where the reliance on local phosphoinositide is dictated by an involvement of Gβγ. Whilst the present study focuses on heterologous expression the findings have physiological implications because Kv7 channel isoforms have a key role in many cell types. Kv7.1 comprises the late repolarising component of the cardiac action potential, Kv7.2/7.3 and Kv7.5/7.3 heteromers constitute the M-channel in neurones and Kv7.4 has a role in cochlear as well as arterial reactivity. Dysregulation of these channels impacts considerably on cellular activity in each cell type under consideration. Defining the mechanisms that regulate Kv7 channels is therefore paramount for understanding physiological and pathophysiological processes. We have already established that βγ G proteins modulate endogenous Kv7 channels in arterial smooth muscle cells consistent with their effects on heterologously expressed Kv7.4 [24] and it is likely that similar effects occur on native Kv7 channels in other systems. If this is the case, then revelation of the present study will have profound resonance for cellular regulation.

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**Conflict of interest.**

The authors have no conflicts of interest.

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**Figure 1. $PIP_2$ depletion reduces Kv7.4 currents.**

A, Examples of whole cell $K^+$ currents from HEK293 Kv7.4 cells evoked by step depolarisation from -60 mV to +20 mV in the absence (a) and presence of 20 µM wortmannin (b). Currents were recorded every 15 s and wortmannin applied after 60 s. Initial current trace shown in black in both panels. Subsequent traces after 5, 10 and 20 min intervals are shown in blue, green and red, correspondingly. Panel (c) shows the mean amplitude of $K^+$ current at +20 mV in the absence (black) and presence of wortmannin (red). Each point is the mean ± s.e.m. of 4 cells. B, Representative traces of Kv7.4 currents evoked by steps from -60 mV to a range of potential (-90 to +40 mV) in control (a) and after depletion of $PIP_2$ by the cells pre-incubation with wortmannin + short (≤ 30 s) application of trypsin (b). The mean data are shown in panel (c) with control (black, n=22), wortmannin alone (green, n=34), wortmannin plus trypsin (red, n=36) and linopirdine (purple, n= 14). C, Example of cell-attached patch recording from HEK293 Kv7.4 cell showing effect of 20 µM wortmannin. Long term trace is shown in panel (a). Representative expanded 1.75 s segments of channel openings taken from panel (a) highlighting channel activity in the absence and presence of wortmannin are shown in panels (b) and (c). Closed state and multiple open states are denoted by C and O1-O6. D, In-cell Western analysis showing that wortmannin, and other known $PIP_2$ inhibitors, decrease global $PIP_2$ level in HEK293 Kv7.4 cells (n=12-23).

**Figure 2. $PIP_2$ depletion prevents activation of whole cell Kv7.4 currents by Gβγ.**

A, Shows representative whole cell currents evoked by depolarisation from -60 mV to +20 mV in Kv7.4 HEK293 cells under control conditions (a) and after incubation in wortmannin (20 µM, b). Right hand panels show cells perfused internally with Gβγ. B,

Mean data for the effect of intracellular perfused active Gβγ on whole cell currents in the absence and presence of 20 μM wortmannin (n=5).

**Figure 3. Gβγ enhancement of Kv7.4 channels in excised patches is prevented by $PIP_2$ depletion.**

A, Representative inside-out patch recording showing stimulatory action of Gβγ. Expanded 1.75 s segments of channel openings in the absence (b) and presence of 2 ng/ml Gβγ (c) are taken from long-term recording (a). Closed state and multiple open states are denoted by C and O1-O2. B, Mean concentration-effect for Gβγ subunits (n=4-7) fitted with a best-fit sigmoidal to the available data. C, Representative inside-out patch recording from cell pre-incubated with wortmannin before (b) and after (c) Gβγ application. Panels (b) and (c) are expanded 2.5 s segments of channel openings taken from long-term recording (a). Closed state and multiple open states are denoted by C and O1-O2. D, Representative inside-out patch recording from cell pre-incubated with wortmannin before (b) and after (c) $PIP_2$ application. Panels (b) and (c) are expanded 1.35 s segments of channel openings taken from long-term recording (a). Closed state and open states are denoted by C and O1.

**Figure 4. Gβγ inhibition abolishes Kv7.4 currents without change in $PIP_2$ levels.**

A, In-cell Western analysis showing influence of Gβγ inhibitors on $PIP_2$ level in HEK293 Kv7.4 cells (n=11-22). B, Mean I-V relationships of HEK293 Kv7.4 currents evoked from holding potential -60 mV before and after application of M201 (50 μM). C, Mean data for the effect for mechanistically different inhibitors of Gβγ on whole cell $K^+$ currents recorded at +20 mV (n=5-7). D, Representative inside-out patch recording showing inhibitory action of 50 μM M201 on single channel activity. Panels (b) and (c) are

representative 1.5 s segments of channel openings before and after drug application taken from long-term recording (a). Closed and open states are denoted by C and O1. E, Representative inside-out patch recording showing inhibitory action of 10 µM Grk2i on single channel activity. Panels (b) and (c) are expanded 2.8 s segments of channel openings before and after drug application taken from long-term recording (a). Closed state and multiple open states are denoted by C and O1-O3. F, Mean apparent open probability for Kv7.4 channels in control conditions (green column, n=14) and after application of three different Gβγ inhibitors, M201, gallein and Grk2i (n=4-6).

**Figure 5. Gβγ inhibition prevents activation of Kv7.4 channels by exogenous $PIP_2$.**

A, Representative inside-out patch recording showing stimulatory action of $PIP_2$. Panels Ab and Ac are 1.75 s segments of channel openings in the absence (b) and presence of $PIP_2$ (100 µM ,c) taken from long-term recording (Aa). Closed state and multiple open states are denoted by C and O1-O3. B, Mean concentration-effect for $PIP_2$ (n=4-11). C, Representative inside-out patch recording showing that in the continued presence of 50 µM M201 (continuation of recording from patch shown in Fig. 4D) 100 µM $PIP_2$ applied to the patch failed to activate the channels. Panel (Cb) is a 1.5 s segment of channel openings taken from long-term recording (Ca). Closed state and multiple open states are denoted by C and O1. D, Representative inside-out patch recording showing that in the continued presence of 100 µM gallein 100 µM $PIP_2$ applied to the patch failed to activate the channels. Panel (Db) is a 2.5 s segment of channel openings taken from long-term recording (Da). E, Representative inside-out patch recording showing that in the continued presence of 100 µM Grk2i (continuation of recording from patch shown in Fig. 4E) 100 µM $PIP_2$ applied to the patch produced only negligible activation of the channels. Panel (Eb) is a 2.8 s segment of channel openings taken from long-term recording (Ea).

Panel F shows the normalised increase in NPo produced by 100 μM $PIP_2$ applied to inside-out patches in the absence and presence of different Gβγ inhibitors (n=4-6).

**Fig 6. $PIP_2$ and Gβγ are synergistic regulators of Kv7.4 channels.**

A, Representative inside-out patch recording showing lack of effect of 1 ng/ml Gβγ on channel activity. Panel (Ab) is an expanded 1.65 s segment of channel openings taken from long-term recording (Aa). Closed and open states are denoted by C and O1. B, Application of 3 μM $PIP_2$ in the presence of 1 ng/ml Gβγ (patch from (A)) significantly increased channel activity. Panel (Bb) is an expanded 1.65 s segment of channel openings taken from long-term recording (Ba). Closed state and multiple open states are denoted by C and O1-O3. C, Mean apparent open probability for Kv7.4 in control conditions (n=16), after application of low concentrations of Gβγ (n=8) and $PIP_2$ alone (n=8) and in combination (n=6). D, Mean apparent open probability for Kv7.4 activated by $PIP_2$ alone (n=4-11, dark blue columns), and in the presence of 1 ng/ml Gβγ (cyan columns, n=4-10).

**Fig 7. Effect of Kv7 channel openers in $PIP_2$- and Gβγ- depleted cells.**

Mean data showing action of Retigabine, S-1 and NS15370 in HEK293 Kv7.4 cells under various conditions (control, pre-incubated with wortmannin alone, pre-incubated with wortmannin + short (≤ 30 s) application of trypsin, pre-incubated with gallein. Currents after treatment with pan-Kv7 channel blocker linopirdine shown for reference. *, **, *** and **** denotes $p<0.05$ - $p<0.0001$ compared to controls.

Figure 1



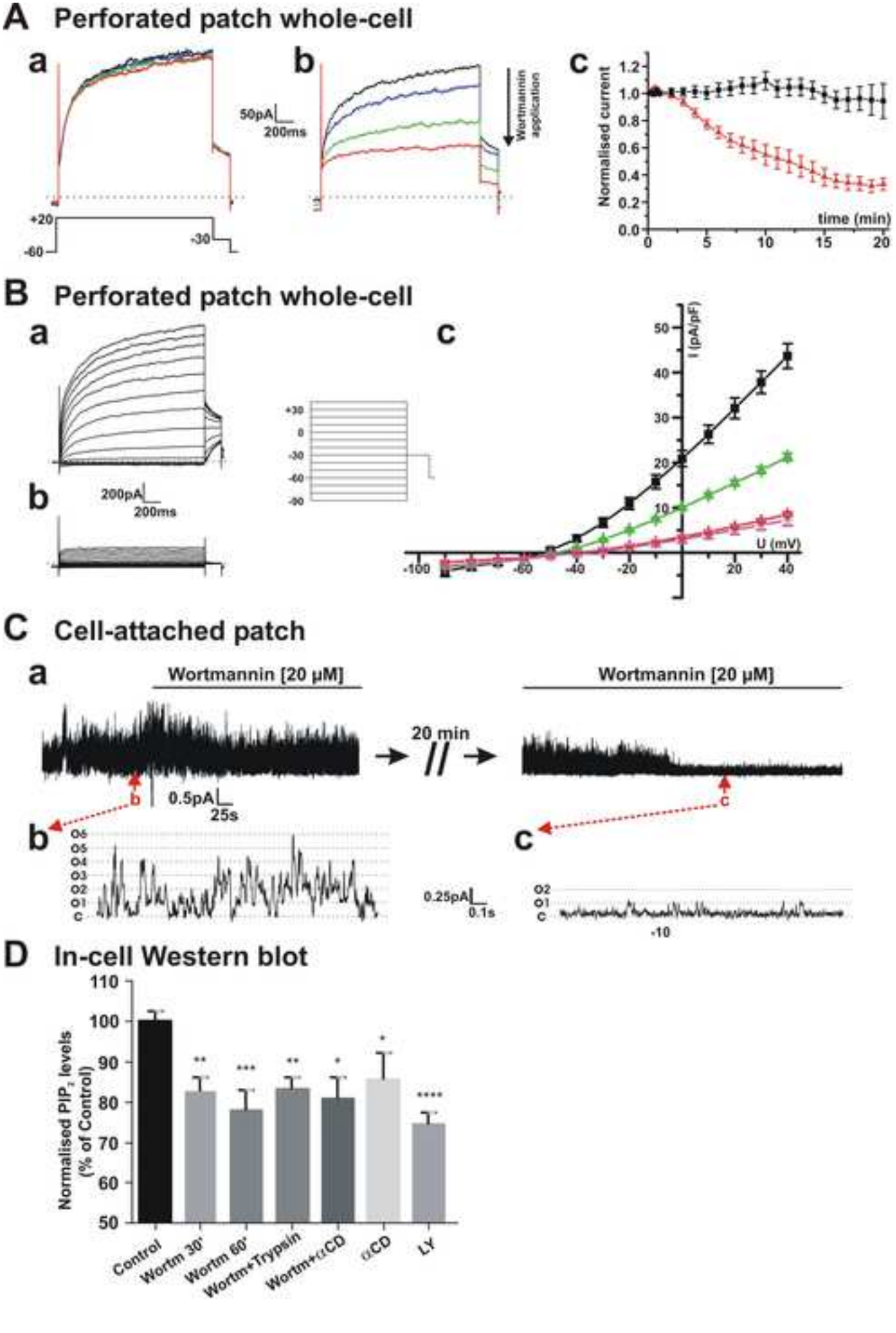

Figure 2



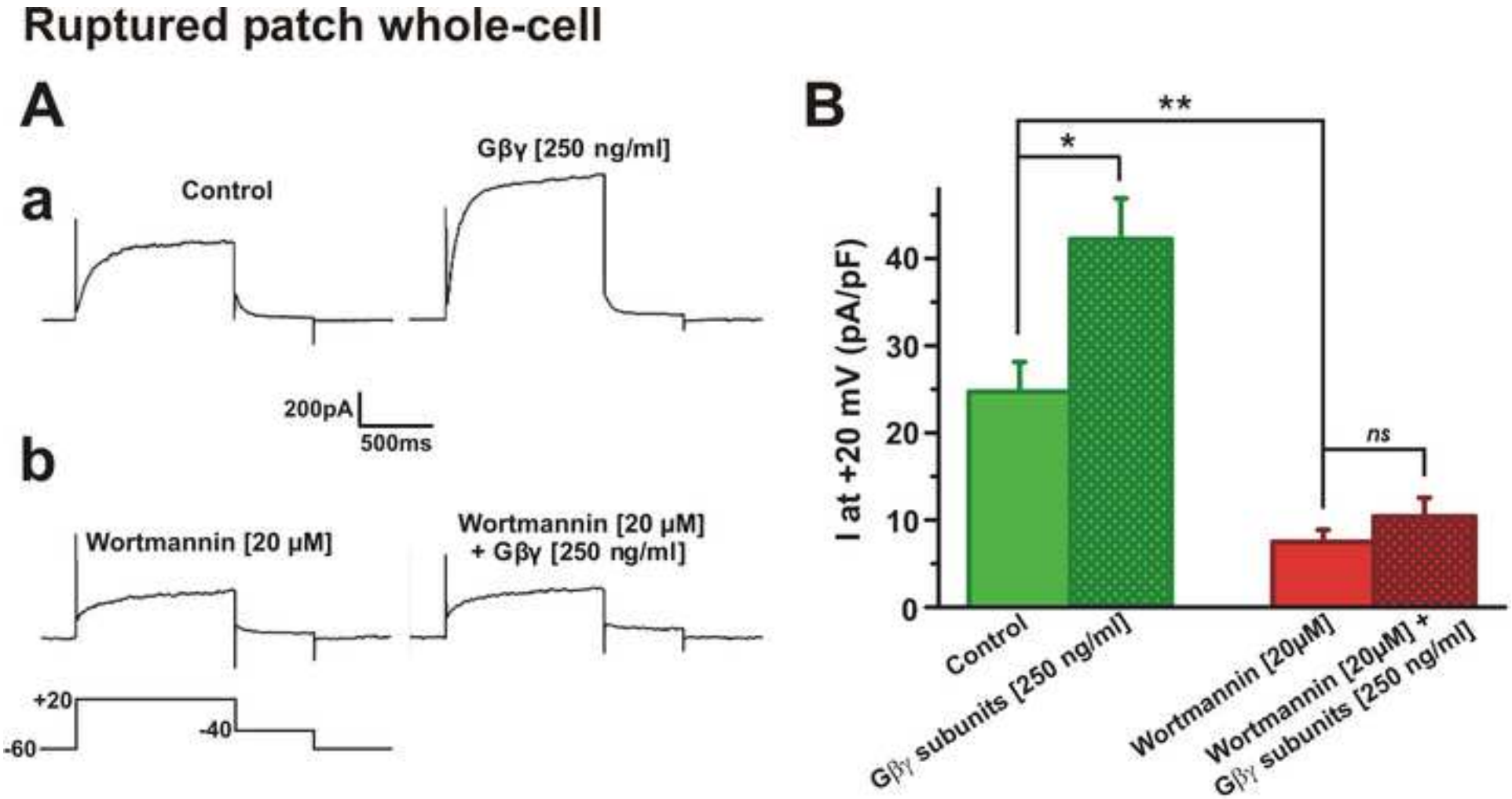

Figure 3



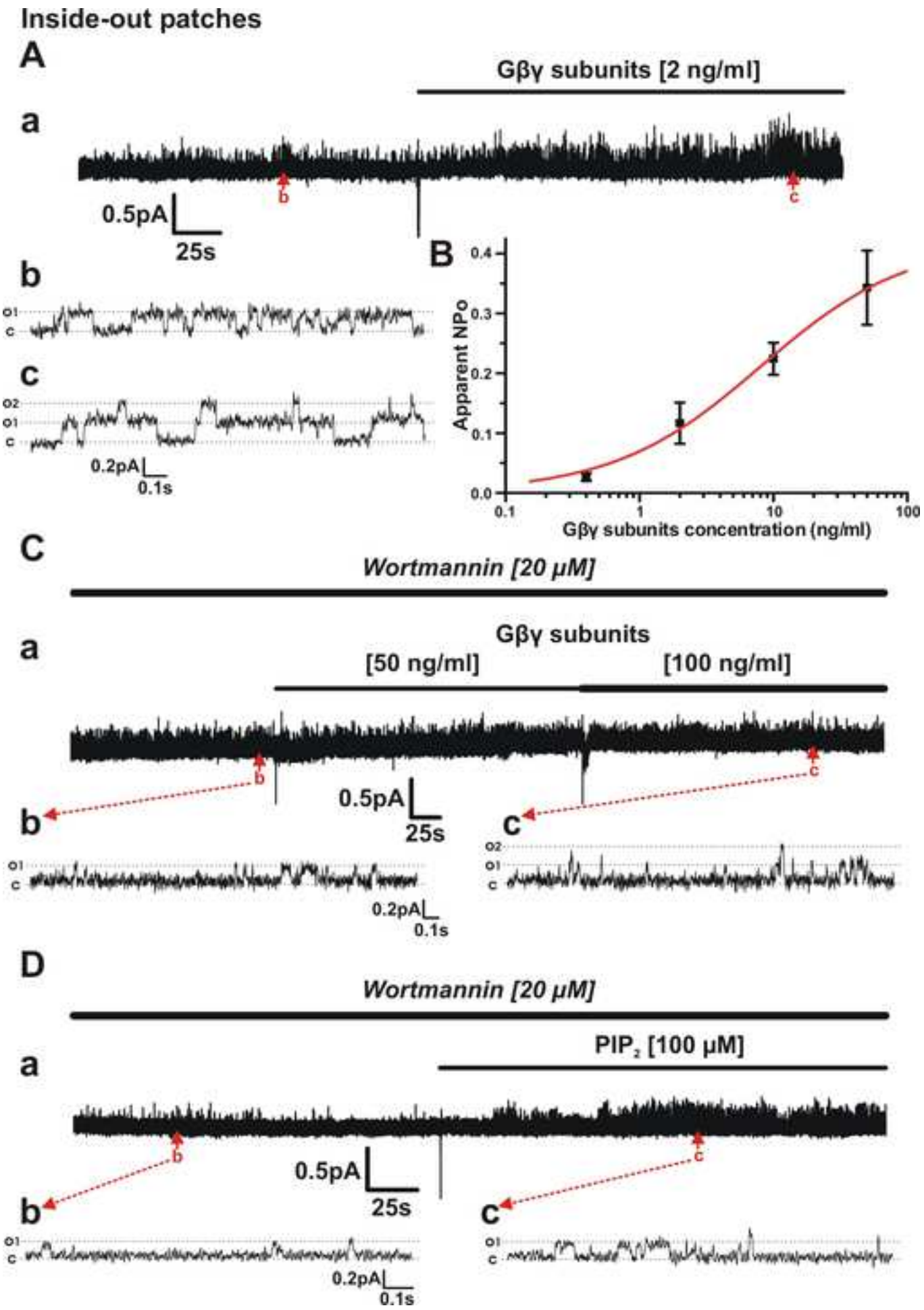

Figure 4

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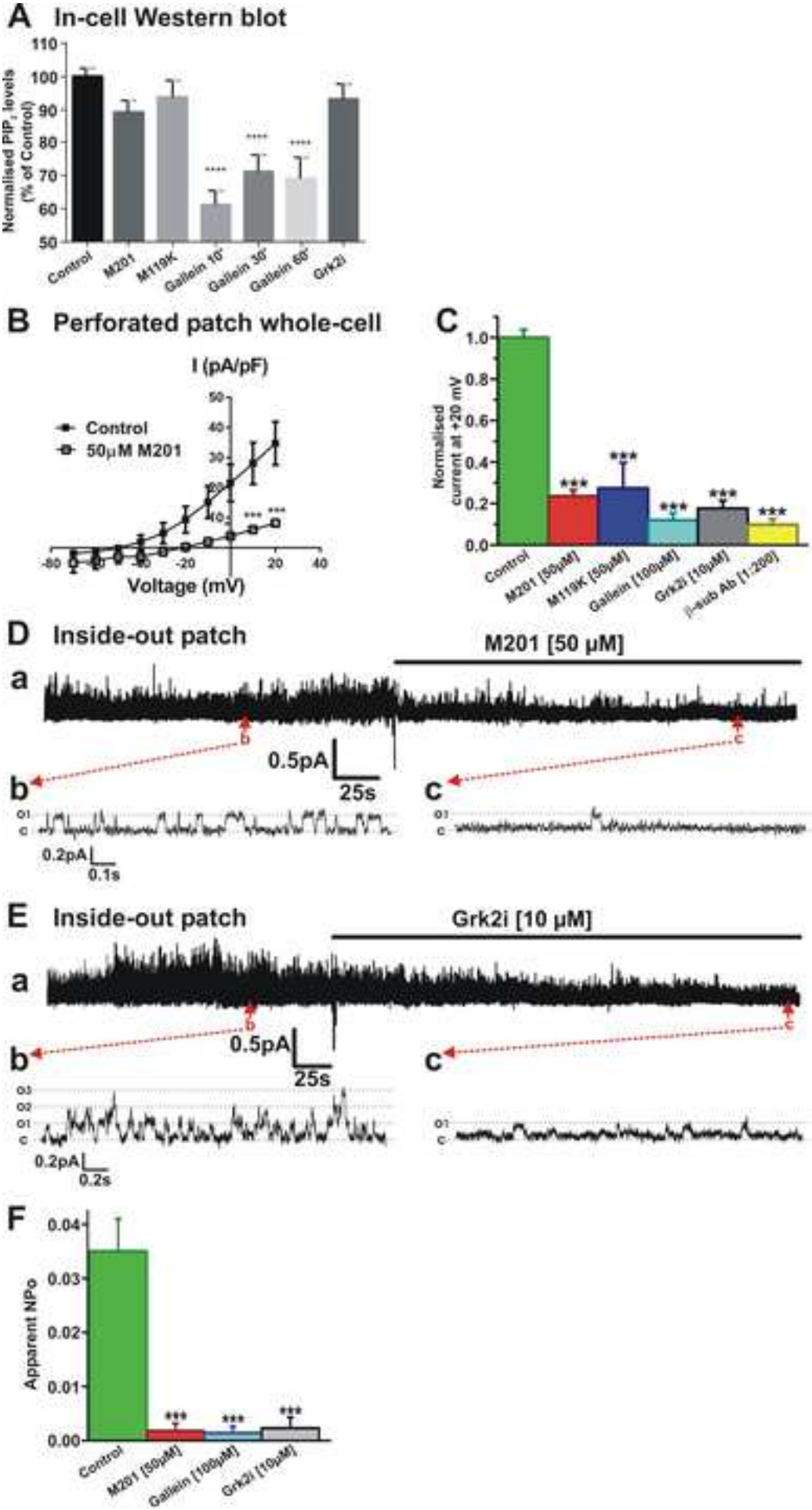

Figure 5



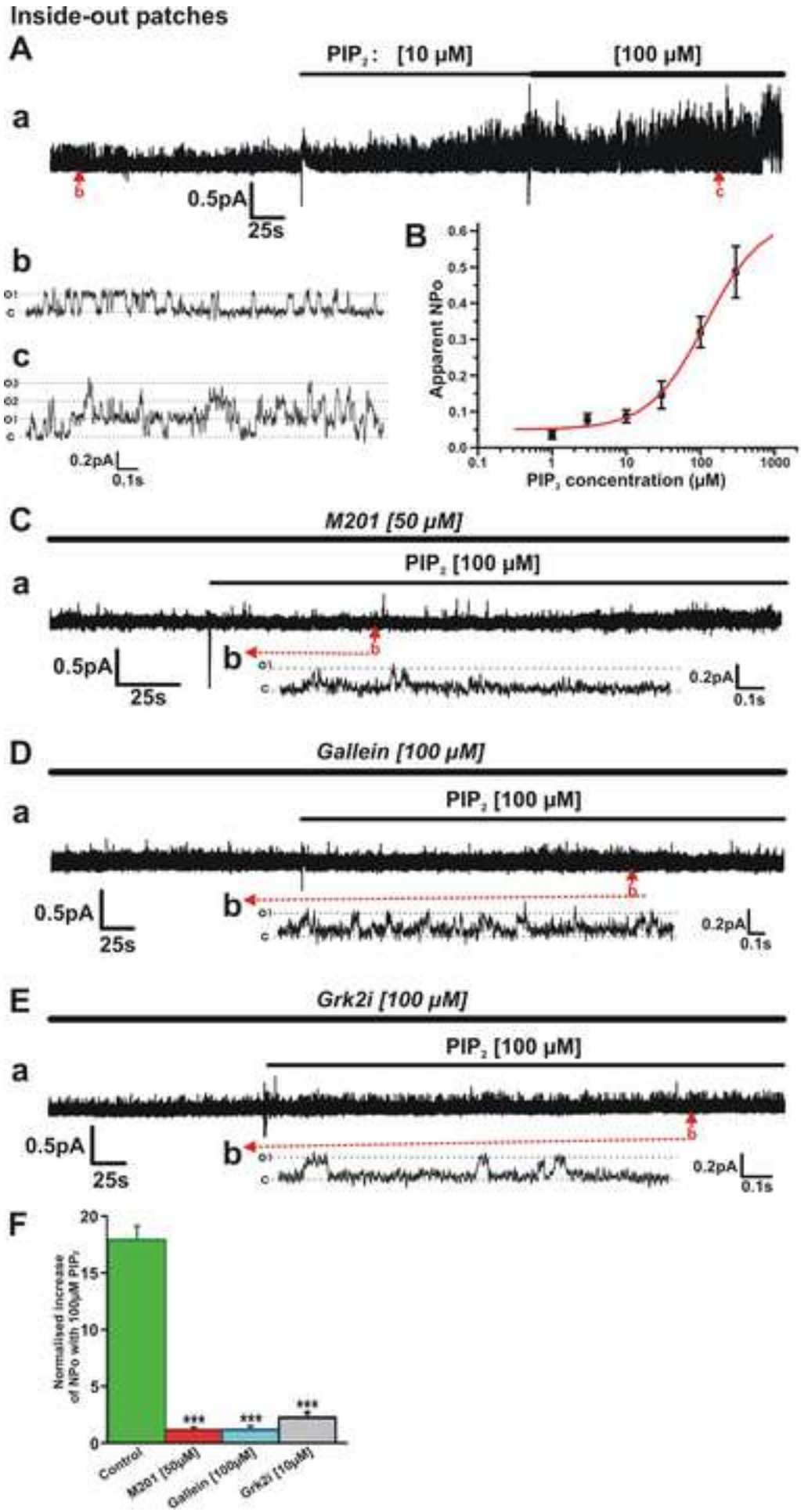

Figure 6

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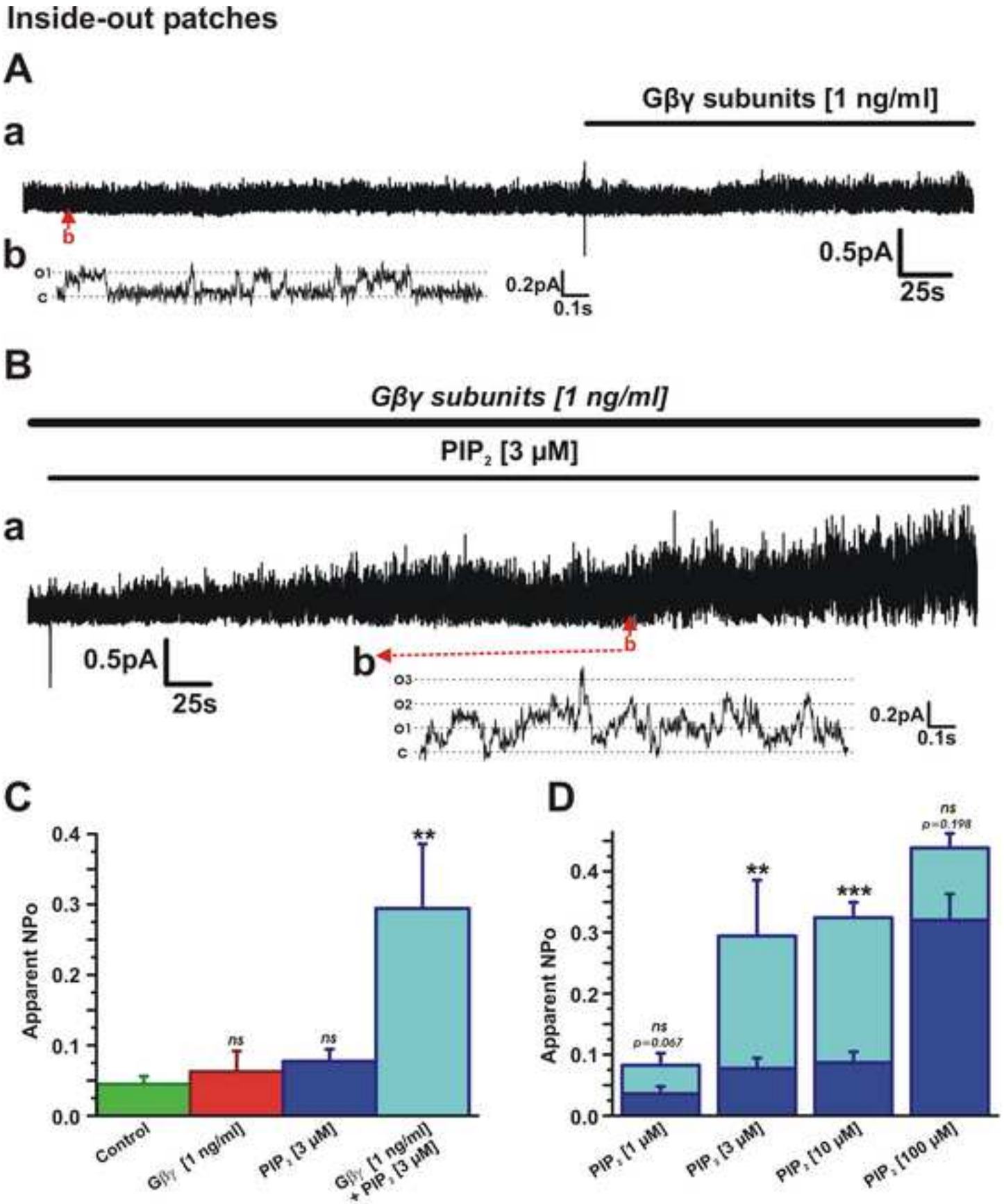

Figure 7

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