

The GB1 and GB3 subunits differentially regulate rat vascular Kv7 channels

Iain A. Greenwood¹, Jennifer B. Stott^{1*}

¹St George's, University of London, United Kingdom

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Author contribution statement

JBS undertook the research, analysed and interpreted the data and wrote the manuscript. JBS and IAG designed and funded the data and revised the manuscript. Both authors read and finally approved the manuscript.

Keywords

G protein subunit, Kv7 channel, vascular smooth muscle, G protein signaling, potassium channel

Abstract

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Within the vasculature Kv7 channels are key regulators of basal tone and contribute to a variety of receptor mediated vasorelaxants. The Kv7.4 isoform, abundant within the vasculature, is key to these processes and was recently shown to have an obligatory requirement of G-protein $\beta\gamma$ subunits for its voltage dependent activity. There is an increasing appreciation that with 5 GB subunits and 12 Gy subunits described in mammalian cells that different GB γ combinations can confer selectivity in GB γ effector stimulation. Therefore, we aimed to characterise the GB subunit(s) which basally regulate Kv7.4 channels and native vascular Kv7 channels. In Chinese Hamster Ovary cells overexpressing Kv7.4 and different GB γ subunits only GB1, GB3 and GB5 enhanced Kv7.4 currents, increasing the activation kinetics and negatively shifting the voltage dependence of activation. In isolated rat renal artery myocytes, proximity ligation assay detected an interaction of Kv7.4 with GB1 and GB3 subunits, but not other isoforms. Morpholino directed knockdown of GB1 in rat renal arteries did not alter Kv7 dependent currents but reduced Kv7.4 protein expression. Knockdown of GB3 in rat renal arteries resulted in decreased basal K⁺ currents which were not sensitive to pharmacological inhibition of Kv7 channels. These studies implicate the GB1 subunit in the synthesis or stability of Kv7.4 proteins, whilst revealing that the GB3 isoform is responsible for the basal activity of Kv7 channels in native rat renal myocytes. These findings demonstrate that different GB subunits have important individual roles in ion channel regulation.

Contribution to the field

This manuscript advances our understanding of the regulation of vascular Kv7 channels by G protein beta gamma subunits. This work reveals that this regulation is not only specific to certain beta subunit isoforms, but also that different isoforms may possess different regulatory properties. This is a considerable conceptual advance in terms of how we think about Kv7 channel regulation in determining that specificity of the isoform subtype is a key feature of channel regulation. This has wide ranging implications for how we think not only about Kv7 channel regulation, but also in the way we think about beta gamma subunit regulation of effectors.

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Data availability statement

Generated Statement: The datasets generated for this study are available on request to the corresponding author.

In review

The G β 1 and G β 3 subunits differentially regulate rat vascular Kv7 channels

Iain A Greenwood¹ and Jennifer B Stott¹

¹Vascular Biology Research Group, Institute of Molecular and Clinical Sciences, St George's University of London, London, UK

Correspondence:

Jennifer B. Stott, PhD

Vascular Research Group

St Georges, University of London, Cranmer Terrace

London, SW17 0RE, UK

Tel: +44 (0) 208 725 6444

Email: jstott@sgul.ac.uk

Keywords: G protein $\beta\gamma$ subunits, Kv7 channels, vascular smooth muscle

Abstract

Within the vasculature Kv7 channels are key regulators of basal tone and contribute to a variety of receptor mediated vasorelaxants. The Kv7.4 isoform, abundant within the vasculature, is key to these processes and was recently shown to have an obligatory requirement of G-protein $\beta\gamma$ subunits for its voltage dependent activity. There is an increasing appreciation that with 5 G β subunits and 12 G γ subunits described in mammalian cells that different G $\beta_x\gamma_x$ combinations can confer selectivity in G $\beta\gamma$ effector stimulation. Therefore, we aimed to characterise the G β subunit(s) which basally regulate Kv7.4 channels and native vascular Kv7 channels. In Chinese Hamster Ovary cells overexpressing Kv7.4 and different G β_x subunits only G β 1, G β 3 and G β 5 enhanced Kv7.4 currents, increasing the activation kinetics and negatively shifting the voltage dependence of activation. In isolated rat renal artery myocytes, proximity ligation assay detected an interaction of Kv7.4 with G β 1 and G β 3 subunits, but not other isoforms. Morpholino directed knockdown of G β 1 in rat renal arteries did not alter Kv7 dependent currents but reduced Kv7.4 protein expression. Knockdown of G β 3 in rat renal arteries resulted in decreased basal K⁺ currents which were not sensitive to pharmacological inhibition of Kv7 channels. These studies implicate the G β 1 subunit in the synthesis or stability of Kv7.4 proteins, whilst revealing that the G β 3 isoform is responsible for the basal activity of Kv7 channels in native rat renal myocytes. These findings demonstrate that different G β subunits have important individual roles in ion channel regulation.

Introduction

The Kv7 family of voltage gated potassium channels are crucial regulators of membrane excitability (Haick and Byron 2016, Barrese et al. 2018). Within the vasculature this role extends itself to regulating cell contractility where the activity of these channels subdues the activity of voltage gated calcium channels and thus the entry of Ca²⁺- the primary contractile

stimulus. This has been evidenced in several vessel types where pharmacological blockade of Kv7 channels results in increased contractility of vessels in *ex vivo* preparations (Chadha et al. 2012, Lee et al. 2015, Stott et al. 2015), and reduced renal blood flow *in vivo* (Salomonsson et al. 2015). Of the 5 Kv7 isoforms (Kv7.1-Kv7.5), the Kv7.4 channel has been most implicated in the regulation of vascular reactivity – molecular knockdown of this isoform increases vessel contractility whilst decreasing the ability to respond to vasodilators (Chadha et al. 2012 and 2014, Stott et al. 2016 and 2018), and it is Kv7.4 expression which is reduced in hypertensive animal models (Jepps et al. 2011). Due to their importance in vascular reactivity, uncovering mechanisms which govern native vascular Kv7 channel activity has been of key interest and recently G protein $\beta\gamma$ subunits ($G\beta\gamma$) were shown to be crucial regulators of basal Kv7 channel activity (Stott et al. 2015). These two strongly bound proteins were originally identified as components of heterotrimeric G proteins, which couple to G protein-coupled receptors (GPCR). However, $G\beta\gamma$ subunits were acknowledged to be important signalling mediators with the discovery that muscarinic acetylcholine induced hyperpolarisation of cardiomyocytes occurred via $G\beta\gamma$ -mediated activation of the K_{ACh} channel (Kir3.1/3.4) (Logothetis et al. 1987). Subsequently it has been shown that $G\beta\gamma$ regulate other ion channels (CaV_2 (Herlitze et al. 1996), TRPM3 (Badheka et al. 2017, Dembla et al. 2017, Quallo et al. 2017) as well as numerous enzymes (e.g. adenylyl cyclase, PI-3 Kinase (Federman et al. 1992, Ford et al. 1998, Stephens et al. 1994).

Proximity ligation assays (PLA) studies with Kv7.4 and $G\beta$ antibodies revealed a high level of channel- $G\beta\gamma$ interaction in unstimulated smooth muscle cells and structurally different inhibitors of $G\beta\gamma$ effector sites (eg gallein, M119K, GRK2i) attenuated heterologously expressed Kv7.4 channels and smooth muscle Kv7 currents in the absence of receptor stimulation (Stott et al. 2015). These findings revealed that Kv7.4 is constitutively regulated by an obligatory interaction with $G\beta\gamma$. However, there are 5 $G\beta$ (1-5) and 12 $G\gamma$ (1-5, 7-13)

subunits described in mammals, and subunits display specificity in forming dimer pairs with the $G\beta_x\gamma_x$ composition known to alter GPCR behaviour and effector coupling (Bayewitch et al. 1998a and 1998b, McIntire et al. 2001, Macrez-Lepretre et al. 1997, Khan et al. 2015). Moreover, there are preferential associations for GPCR-ion channel couplings e.g. $G\beta\gamma$ inhibition of N-type calcium channels after α_2 -adrenoceptor stimulation is more effective when the receptor is coupled with $G\beta_1$ or $G\beta_2$ (Mahmoud et al. 2012), whilst β_1 -adrenoceptor coupling to Kir3.2 displays a preference for $G\beta_5$ containing dimers (Robillard et al. 2000). We aimed to determine if a specific $G\beta$ subunit was responsible for the basal regulation of Kv7.4 in native arterial smooth muscle cells and ascertain the effect of the 5 different $G\beta$ isoforms on heterologously expressed Kv7.4. Our data reveal a striking difference between different $G\beta$ isoforms that impacts on vascular responsiveness.

Results

Stott et al. 2015 showed that intracellular perfusion of heterogeneous $G\beta\gamma$ subunits isolated from bovine brain enhances heterologously expressed Kv7.4 currents, produces a leftward shift in the voltage dependence of activation and reduces the rate of activation of these currents. These findings were replicated in CHO cells transiently transfected with Kv7.4, where intracellular perfusion with 250ng/ml of $G\beta\gamma$ subunits significantly increased voltage dependent currents compared with control (**FIG 1**). $G\beta_1$ -4 show a high degree of homology, whereas $G\beta_5$ is the most structurally distinct $G\beta$ subunit. To determine the effect of individual $G\beta$ subunits from the structurally similar $G\beta_1$ -4 group on Kv7.4 currents, we examined the effect each of these subunits on heterologously expressed Kv7.4 channels. Chinese Hamster Ovary (CHO) cells transfected with both Kv7.4 and $G\beta_1$ or $G\beta_3$ plasmids significantly increased K^+ currents compared to cells expressing Kv7.4 and the empty vector (e.g. 12.4 ± 1.6

pA/pF to 27.9 ± 7.7 (G β 1) and 22.5 ± 4.2 (G β 3) at 40mV) (**FIG. 2B and C**). For both G β subunits this effect on Kv7.4 was accompanied by a leftward shift in the voltage dependence of activation (from -2.3mV to -9.1mV (G β 1) and -5.9mV (G β 3)) (**FIG. 2D**) and an increase in the rate of activation of the currents over a range of voltages (from 576 ± 45.4 ms to 316.7 ± 53.7 ms (G β 1) and 310 ± 49.2 ms (G β 3) at 40mV) (**FIG. 2E**). These effects on kinetics are analogous to the effect of G $\beta\gamma$ subunits isolated from bovine brain on Kv7.4 (Stott et al. 2015). In contrast, neither G β 2 nor G β 4 affected heterologously expressed Kv7.4 currents (11.9 ± 3.8 pA/pF (G β 2) and 14.4 ± 5.7 pA/pF (G β 4) at 40mV) (**FIG. 3A and 3B**). Interestingly, however, the structurally distinct G β 5 also significantly enhanced Kv7.4 currents (26.3 ± 6.8 pA/pF at 40mV), produced a leftward shift in the voltage dependence of activation (-8.1mV) and increased the rate of activation of the currents (319 ± 34 ms) (**FIG. 3C-E**).

To uncover the G β x subunit(s) responsible for the basal activity of the native vascular Kv7 channel, we first examined the expression of the three G β isoforms which enhanced Kv7.4 currents - G β 1, 3 and 5. Immunofluorescence performed on isolated rat renal artery myocytes showed strong expression of both G β 1 and 3 in the cytosolic and sub-membranous regions (**FIG. 4A and B**). Interestingly, expression of G β 5 was confined to the nucleus of these cells (**FIG. 4C**). To ascertain which subunits interacted with Kv7.4 in rat renal myocytes we further undertook a series of Proximity Ligation Assay (PLA) studies. It has been shown that proteins can influence ion channels from distances ≤ 200 nm (Tajada et al. 2017), and PLA detects protein-protein interactions well within this range at ≤ 40 nm. PLA was performed in rat renal artery myocytes either in control or after treatment with 50 μ mol/L gallein. Gallein disrupts G $\beta\gamma$ interactions and so was used as a pharmacological control in determining positive interactions. PLA punctae detected for the combinations of both Kv7.4-G β 1 (8.2 ± 0.8 puncta/cell) and Kv7.4-G β 3 (18.7 ± 2 puncta/cell) which were significantly reduced by gallein treatment (change to 3.8 ± 0.4 (G β 1) and 8.8 ± 1.8 (G β 3) puncta/cell) (**FIG 5**). PLA experiments

with Kv7.4-G β 5 PLA antibody combinations did not detect any gallein sensitive PLA puncta (2.7 ± 0.8 puncta/cell to 2.0 ± 0.4 puncta/cell) (**FIG. 5**). These data reveal an association of Kv7.4 with G β 1 and G β 3 in vascular smooth muscle cells.

In order to assess the role of G β 1 and G β 3 on native vascular Kv7 currents, we performed morpholino directed knockdown of each subunit and examined the Kv7 currents by electrophysiology. Control cells produced K⁺ currents which had a modest sensitivity to the Kv7 channel blocker linopirdine (10 μ mol/L) with currents at +40mV being reduced from 7.2 ± 0.8 pA/pF to 4.2 ± 0.4 pA/pF (**FIG. 6A**). G β 1 morpholino knockdown cells produced K⁺ currents of the same magnitude as control (6.41 ± 1 pA/pF at 40mV), whereas K⁺ currents in G β 3 morpholino knockdown cells were significantly reduced (3.8 ± 0.7 pA/pF at 40mV) (**FIG. 6B**). Kv7 inhibition with 10 μ mol/L linopirdine significantly reduced K⁺ currents in G β 1 cells (from 6.41 ± 1 pA/pF to 3.2 ± 0.6 pA/pF at 40mV) (**FIG. 7A**). Immunofluorescence confirmed that G β 1 morpholino knockdown reduced G β 1 expression (**FIG. 7B**), and did not affect G β 3 expression (**FIG. 7C**). However, total Kv7.4 abundance was also reduced in G β 1 knockdown cells (**FIG. 7D**). In cells treated with G β 3 morpholino, 10 μ mol/L linopirdine had no significant effect on K⁺ currents (from 3.8 ± 0.7 pA/pF to 2.8 ± 0.4 pA/pF at 40mV) (**FIG. 8A**). Immunofluorescence confirmed knockdown of G β 3 (**FIG. 8B**), whilst no effects on G β 1 or Kv7.4 expression was seen (**FIG. 8C and 8D**).

Discussion

Several ion channels have now been shown to be regulated by G $\beta\gamma$ subunits revealing the importance of G $\beta\gamma$ subunit-channel regulation in different physiological processes. However, with the increasing recognition of the importance of individual G $\beta_x\gamma_x$ combinations in conferring selectivity in effector regulation, defining these properties is the new frontier in G $\beta\gamma$ research. This study demonstrates for the first time the importance of individual G β subunits

on Kv7.4 channel function. We show that Kv7.4 channel activity can be regulated by several G β isoforms in a cell expression system (G β 1, 3 and 5), but that in native vascular myocytes specific roles for different subunits are described. G β 1 subunits appear to regulate the synthesis or stability of Kv7.4, whereas G β 3 regulate basal channel activity in rat renal artery myocytes.

G $\beta\gamma$ specificity

With 5 G β (1-5) and 12 G γ (1-5, 7-13) subtypes expressed in mammals, a growing body of evidence has demonstrated specific functions for different subunits, but it remains an oft overlooked aspect of G $\beta\gamma$ regulation. G β subunits are highly homologous and between G β 1, 2, 3 and 4 there are 79-90% sequence similarities (Khan et al. 2013). G β 5 is the least homologous and shows 51-52% homology when compared to all other subtypes. However, different subunits clearly exert different regulations and here we show specificity in the G β isoforms which can regulate Kv7.4 and native vascular Kv7 channels. Only G β 1, 3 and 5 mirrored the effect on Kv7.4 currents previously reported for purified G $\beta\gamma$ (i.e. increased current amplitude, leftward shift in voltage dependence of activation and increased rate of activation) (Stott et al. 2015). G β 2 and 4 were without effect. Of the 3 G β isoforms that enhanced Kv7.4 channels, only G β 1 and 3 interacted with Kv7.4 in a gallein-dependent manner in renal artery myocytes. Moreover, only G β 3 knockdown impaired native Kv7 channel currents analogous to treatment with gallein reported previously (Stott et al., 2015). It is interesting that although G β 1 enhanced Kv7.4 currents in an overexpression system, knockdown of G β 1 had no effect on Kv7 currents in renal artery myocytes even though there is a decrease in Kv7.4 protein in these cells. It is likely that the reduction in Kv7.4 is not severe enough to affect overall current density in the acute knockdown protocol over 48 hours that we performed. However, our findings clearly indicate that G β 1 regulates the expression of Kv7.4 at some level (discussed in detail below). Overall the results from our expression and native studies suggest that even though G β 1 can

regulate Kv7.4 currents, in myocytes that role is played by G β 3 and this could indicate that G β 3 is the preferential regulator of Kv7.4.

Another consideration is that native Kv7 channels present in rat renal artery myocytes could well be a hetero-tetramer with Kv7.5 subunits (Chadha et al. 2014, Brueggemann et al. 2014), and currents produced by these hetero-tetramers are also enhanced with application of G β γ isolated from bovine brain (unpublished data). It is unclear if differential regulation by different G β seen here for Kv7.4 would also apply to the Kv7.4/7.5 channel although it seems likely. It is also known that ~~and are likely complexed~~ vascular Kv7 channels undergo regulation by other molecules ~~with~~ e.g. KCNE subunits (Jepps et al. 2015). It is possible that in the native system where such complexities exist, these different factors contribute to the G β regulation of the channel. Further studies will investigate how different regulators and channel compositions alter G β subunit regulation, but it is clear from our study is that G β 3 basally regulates the native Kv7 current in renal artery myocytes. This is of interest in the area of the vascular biology of hypertension, where a polymorphism in G β 3 has been associated with a hypertensive phenotype (Semplicini et al. 2015). This polymorphism produces a truncated version of the G β 3 protein, but it remains to be seen how this might regulate Kv7.4 or native vascular Kv7 channels. Finally, the role of G β 5 remains unclear as we did not find this to be associated with Kv7.4 in rat renal artery myocytes, but it is possible that this may be an important regulator of Kv7.4 channels in other cell types.

It is important to note that our study has not investigated the role of G γ in Kv7.4 channel regulation and this could well be an important determinant as described recently for Kir channels (Tabak et al. 2019). Certainly, in overexpression studies the G γ natively expressed in these cells could be a determinant in the behaviour of the G β subunits expressed. Subunits have been shown to exhibit preferences in forming G β γ _x dimers (Pronin and Gautam 1992, Schmidt et al. 1992, Dupre et al. 2009) whereas G β 5 is thought to preferentially couple not to G γ , but

to a Regulator of G protein Signalling (RGS7) (Cabrera et al. 1998). There have also been differing reports about the coupling of G β 3, with some reports showing that it can function without G γ (Schmidt et al. 1992, Dingus et al. 2005 and Poon et al. 2009~~Badheka et al. 2017~~). This is likely due to differences in experimental methods used, but what is clear is that what can theoretically interact *in vitro* may not be what actually occurs *in situ*. G γ subunits are much more diverse, and some have argued that the diversity in G $\beta\gamma$ signalling is due to the G γ in the dimer, rather than the G β . However our studies are complemented by work in native cells and we clearly show definitive roles for G β 1 and 3 here and we demonstrate that disabling this subunit affects Kv7 channels. However, further studies will begin to examine the role of G γ in these processes.

G $\beta\gamma$ in trafficking

We describe here that knockdown of G β 1 decreases Kv7.4 abundance in renal artery myocytes. This was an unexpected finding from a control experiment, but displays another different facet of G β regulation. As discussed above, the decrease in Kv7.4 was not severe enough to affect Kv7 current density, but it is possible that a longer period of knockdown would give more information on this relationship. Knockout of G β 1 has been shown to be embryonic or perinatally lethal so this is not possible (Okoe and Iwakura 2010). However, these findings are supported by previous findings where G β 1 regulate protein expression - knockdown increases G β 4 expression (Hwang et al. 2005) and also alters Kir expression levels (Zylbergold et al. 2014). As it stands it is impossible to know at what level G β 1 regulates Kv7.4 expression in myocytes without a much more detailed investigation into Kv7.4 protein synthesis and stability in these cells, but insights from previous work shows us that G $\beta\gamma$ can variously be involved in transcription, translation, trafficking and degradation of proteins and their genes (Khan et al. 2016). Knockdown studies have also demonstrated that G β subunits are involved in the expression of G γ subunits - G β 1 knockdown results in decreased G γ 5 expression, whilst G γ 2,

5 and 12 were decreased by G β 2 knockdown (Hwang et al. 2005) – but this may be not be a direct regulatory effect but due to a result of removing the binding partners of these G γ subunits ceases their production.

These findings underlie how the identity of the G β subunit is crucial for different aspects of ion channel regulation. That we demonstrate that two closely related G β subunits have two different regulatory capacities for Kv7.4 channels in the vasculature is quite striking, and is important in changing the way we think about G $\beta\gamma$ regulation. This work is significant in the growing move away from viewing G $\beta\gamma$ as homogenous dimer pairs to viewing these subunits as complex, disparate entities with specific regulatory responsibilities within cells.

Materials and Methods

Ethical Approval

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986

Cell Culture Chinese Hamster Ovary (CHO) cells were maintained in Dulbecco's modified Eagle's medium – high glucose solution supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin in an incubator with 5% CO₂. Cells were split into 6 well plates for transfection for electrophysiology experiments. Cells were transfected with Kv7.4 and either G β 1-5 or an empty vector (EV). Plasmids were mixed with Lipofectamine 200 in Opti-MEM for 20 mins before being added to cells. A total of 3 μ g of plasmid was transfected in a 1:1 ratio for 24 hours. Cells were briefly trypsinised on the day of experiments and plated on 13mm coverslips in media at room temperature for 30mins and were then stored in the fridge for use within 8 hours.

Animals Male Wistar rats (175-225g, Charles River UK) were kept in a 12hr light/dark cycle with free access to food and water. Animals were culled on the day of experiment by Schedule

1 cervical dislocation. Renal arteries (RA) were dissected of adherent fat and connective tissue and stored on ice in a physiological saline solution (PSS) containing (in mmol/L); 4.5 KCl, 120 NaCl, 1.2 MgSO₄·7H₂O, 1.2 NaH₂PO₄·2H₂O, 25 NaHCO₃ 5 D-Glucose and 1.25 CaCl₂.

Morpholino Knockdown To study the role of Gβ1 and Gβ3 in native Kv7 currents, knockdown in RA was performed by transfection with morpholino nucleotides as described in vessels previously (Stott et al. 2018). Gβ1 and Gβ3 morpholino nucleotides and mismatched control nucleotides (5 μmol/L, Genetools) were mixed with Lipofectamine 2000 (Life Technologies, UK) in Opti-MEM and left at room temperature for 2 hours. RA were transfected with this mix in Dulbeccos modified eagle's medium F-12 with 1% penicillin/streptomycin at 37°C for 48 hours.

Cell Isolation Freshly dissected renal arteries were used for isolation of individual myocytes for proximity ligation assay, whereas vessels incubated in control or morpholino for 48 hours were used for electrophysiology. Vessels were bathed for 10 minutes in a nominally Ca²⁺ free solution (in mmol/L: 6 KCl, 120 NaCl, 1.2 MgCl₂, 12 D-glucose and 10 HEPES, pH 7.4 with NaOH). Vessels were then incubated at 37°C for 20 (incubated) or 23 (fresh) mins in Ca²⁺ free solution containing in mg/ml: 1.5 collagenase, 0.75 thermolysin, 1 trypsin inhibitor and 1 bovine serum albumin (all Sigma Aldrich, UK). Vessels were then washed in Ca²⁺ free solution for 10 mins and then triturated to liberate myocytes. The cell solution was plated on 13mm coverslips in a 24 well plate and supplemented with an equivalent volume of 2.5mmol/L Ca²⁺ solution to allow the cells to adhere.

Immunofluorescence Freshly isolated myocytes from renal arteries from vessels transfected with control or Gβ1 and Gβ3 morpholino were prepared as above. Cells were fixed with 3% PFA on ice for 20 mins and stored in PBS at 4°C. Cells were treated with 0.1mol/L glycine for 5 min and then incubated in blocking solution (PBS containing 0.1% Triton X-100 and 1%

bovine serum albumin) for 1h at RT before being incubated overnight at 4°C with the following primary antibodies: (i) rabbit anti-Kv7.4 antibody (dilution 1:100, AbCam, Cambridge, UK); (ii) rabbit anti-Gβ1(dilution 1:100, Genetex, #GTX114442); mouse anti-Gβ3 (dilution 1:100, Abnova #H00002784-MO1) (iii) rabbit anti- Gβ5 (dilution 1:200, Abcam, #ab185206). Samples were then washed with PBS and incubated for 1h with donkey anti-rabbit or donkey anti-mouse secondary antibodies conjugated to Alexa Fluor 568, respectively (dilution 1:100, Thermo-Fisher, Paisley, UK). All antibodies were diluted in blocking solution. Subsequently, samples were washed with PBS and mounted using Vectashield Antifade Medium containing DAPI for nuclei counterstaining (Vector Laboratories, Peterborough, UK). Coverslips were analysed using a Zeiss LSM 510 Meta argon/krypton laser scanning confocal microscope (Carl Zeiss, Jena, Germany). Corrected total cell fluorescence (CTCF) was calculated using ImageJ software as elsewhere described (Burgess et al. 2010).

Proximity Ligation Assay Freshly isolated cells were used as described above. 1mL of solution containing 2.5mmol/L CaCl₂ was added to each well and cells were placed in an incubator (37°C, 5% CO₂) for 30 minutes to equilibrate. Cells then underwent treatment: 50µmol/L gallein or DMSO control (30mins). Gallein was used as a pharmacological control for identifying positive interactions with Gβx subunits, and only interactions which were sensitive to gallein were considered as true interactions. Coverslips were immediately fixed with 3% PFA on ice for 20 mins and stored in PBS at 4°C. For the proximity ligation assay, cells were permeabilised with 0.01% Triton X for 5 mins. The Duolink in situ PLA detection kit (Sigma-Aldrich, UK) was used to detect single molecule interactions for Kv7.4 (anti-mouse (N43/6, RRID: AB_2131828, UC Davis/NIH NeuroMab Facility) or anti-rabbit (Abcam, Cambridge UK) and G protein β subunits; anti-Gβ1 (rabbit, Genetex, #GTX114442), anti-Gβ2 (rabbit, Cusabio, #CSB-PA009604ESR2HU), anti-Gβ3 (mouse, Abnova #H00002784-MO1) and anti-Gβ5 (rabbit, Abcam, #ab185206). Experiments were performed as per manufacturer's

instructions; primary antibodies were incubated at 1:200 overnight at 4°C. Red fluorescent oligonucleotides produced as the end product of the procedure were visualised using a Zeiss Confocal LSM 510. Images were analysed using Image J software using the particle detector tool. The number of puncta per cell was calculated as the average of two mid sections in each cell.

Electrophysiology All current recordings were made using AXOpatch 200B amplifier (Axon Instruments) at room temperature. Whole –cell electrical signals were generated and digitized at 1kHz using a Digidata 1322A hosted by a PC running pClamp 9.0 software (Molecular Devices). For recordings cells were placed in an external solution containing (in mmol/L); 140 NaCl, 4 KCl, 2 CaCl, 1 MgCl₂ and 10 HEPES. Patch pipettes with a resistance of 4-12 MΩ were filled with a pipette solution containing (in mmol/L); 110 K gluconate, 30 KCl, 0.5 MgCl₂, 5 HEPES and 0.5 EGTA. Cells were held at -60mV and currents amplitude was monitored by application of a test pulse to 40mV every 20s. To generate current-voltage relationships a voltage step protocol was used from a holding potential of -60mV testing a range of voltages from -70 to 40mV in 10mv increments at 15s intervals.

Reagents: Gβ1 plasmid was kindly gifted by Dr Kim Jonas. Gβ2-5 were purchased from Addgene (catalogue nos. 36113, 36114, 54469 and 54470). Gallein and linopirdine were obtained from Tocris (Bristol, UK). Gβγ subunits isolated from bovine brain were purchased from MerckMillipore.

Statistics

All values are expressed as mean ±SEM. All statistical analyses and non-linear regression curve fitting were performed with GraphPad Prism 7 software. In experiments which utilise native rat myocytes ‘n’ represents the number of cells analysed and ‘N’ represents the number of subjects. Analyses of immunofluorescence data were performed by Student’s t-test, and

Proximity Ligation Assay data by one-way ANOVA multiple comparisons test with Bonferroni post-hoc analysis. Analyses of electrophysiological data used two-way ANOVA with Bonferroni post-hoc analysis. Differences were considered significant at $P < 0.05$.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

JBS undertook the research, analysed and interpreted the data and wrote the manuscript. JBS and IAG designed and funded the data and revised the manuscript. Both authors read and finally approved the manuscript.

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Legends to Figures

Figure 1. $G\beta\gamma$ enhances Kv7.4 currents in CHO expression system

Top panels show representative traces of currents generated in cells expressing Kv7.4 either with the control intracellular solution (left) or with 250ng/ml $G\beta\gamma$ purified from bovine brain (right). Zero current is indicated by the dashed line. Mean IV relationship of Kv7.4 currents in control (n=7) or with 250ng/ml $G\beta\gamma$ (n=4) is shown in bottom panel. Data was analysed by

Bonferroni post-hoc test following a two-way ANOVA. $p < 0.01$ is denoted (**) and $p < 0.005$ is denoted (***)

Figure 2. G β 1 and G β 3 positively modulate Kv7.4 currents

(Ai) Diagram of voltage step protocol used to examine IV relationships. (Aii – iv) Representative traces of currents generated in cells expressing Kv7.4 with an empty vector (EV) (ii), G β 1 (iii) or G β 3 (iv). Zero current is indicated by the dashed line and point used for determination of voltage dependence of activation is indicated by the arrow. Mean data is shown in B (G β 1, n=14) and C (G β 3, n=13). (D) Voltage dependence of activation_ to determine V_{1/2} of Kv7.4 with EV (-2.4±2.3mV), G β 1 (-9.12±3.9mV) and G β 3 (-5.9±3.3mV). (E) Time dependence of activation of Kv7.4 currents when expressed with EV, G β 1 and G β 3 from -20mV to 40mV. Data was analysed by Bonferroni post-hoc test following a two-way ANOVA. $p < 0.05$ is denoted (*), $p < 0.01$ is denoted (**) and $p < 0.005$ is denoted (***)

Figure 3. G β 2, G β 4 and G β 5 effects on Kv7.4 currents

IV relationships of Kv7.4 currents in empty vector (EV) or when co-transfected with G β 2 (A, n=12), G β 4 (B, n=10) or G β 5 (C, n=12). (D) Representative traces of currents cells expression Kv7.4+EV (upper panel) or with G β 5 (lower panel). Zero current is indicated by the dashed line and point used for determination of voltage dependence of activation is indicated by the arrow. (E) Voltage dependence of activation curves to determine V_{1/2} of Kv7.4 with EV (-2.4±2.3mV) and G β 5 (-8.1±3mV). (E) Time dependence of activation of Kv7.4 currents when expressed with EV or G β 5 from -20mV to 40mV. Data was analysed by Bonferroni post-hoc test following a two-way ANOVA. $p < 0.05$ is denoted (*), $p < 0.01$ is denoted (**) and $p < 0.005$ is denoted (***)

Figure 4. Immunofluorescence of specific G β subunits in rat renal artery myocytes

Representative images showing staining in isolated rat renal artery myocytes of G β 1 (A), G β 3 (B) and G β 5 (C), all in red pseudocolour. Nuclear staining by DAPI is shown in blue pseudocolour, while membrane staining by Wheat Germ Agglutinin (WGA) is shown in green pseudocolour. Scale bar: 10 μ m.

Figure 5. Proximity Ligation Assay detection of Kv7.4 interactions with specific G β subunits

(A) Representative images of renal artery myocytes investigated for interactions between Kv7.4 and G β 1, G β 3 or G β 5 in control (upper panels) or after treatment with 50 μ mol/L gallein (lower panels). (B) Mean data of number of PLA puncta/cell before and after 50 μ mol/L gallein treatment for Kv7.4- G β 1 (i, n=30-31, N=5), Kv7.4- G β 3 (iii, n=15-33, N=3-4) and Kv7.4- G β 1 (iv, n=11-12, N=2). 'n'=number of cells and 'N' = number of animals. Scale bars represent 10 μ m. PLA data was analysed by one-way ANOVA with Bonferroni post hoc analysis where p<0.01 is denoted (**) and p<0.005 is denoted (***)

Figure 6. K⁺ currents in renal artery myocytes – effects of linopirdine and β 1 or β 3 morpholino

(A) Representative images of whole cell K⁺ currents recorded in rat renal artery myocytes in control (i) or in the presence of 10 μ m/L linopirdine (ii), and mean data (iii, n=17 N=4 (control), n=9 N=4 (linopirdine)). Zero current is indicated by the dashed line. (B) Representative images of whole cell K⁺ currents recorded in rat renal artery myocytes after morpholino knockdown of G β 1 (i) or G β 3 (ii) and mean data (iii, n=10 N=4 (G β 1), n=8 N=4 (G β 3)) Zero current is indicated by the dashed line. 'n'=number of cells and 'N' = number of animals. Data was analysed by Bonferroni post-hoc test following a two-way ANOVA. p<0.05 is denoted (*), p<0.01 is denoted (**) and p<0.005 is denoted (***)

Figure 7. G β 1 knockdown effects on renal artery myocytes

(A) Mean data of whole cell K⁺ currents recorded in rat renal artery myocytes in Gβ1 morpholino in control (n=10, N=4) or in the presence of 10μm/L linopirdine (n=8, N=4). Mean fluorescence intensity of (B) Gβ1 (n=20-22, N=4) (C) Gβ3 (n=15, N=3) or (D) Kv7.4 (n=14, N=3) in renal artery myocytes in control morpholino or Gβ1 morpholino. 'n'=number of cells and 'N' = number of animals. Electrophysiology data was analysed by Bonferroni post-hoc test following a two-way ANOVA. IF data was analysed by one-way ANOVA with Bonferroni post hoc analysis. p<0.01 is denoted (**) and p<0.005 is denoted (***)

Figure 8. Gβ3 knockdown effects on renal artery myocytes

(A) Mean data of whole cell K⁺ currents recorded in rat renal artery myocytes in Gβ3 morpholino in control (n=8, N=4) or in the presence of 10μm/L linopirdine (n=5, N=4). Mean fluorescence intensity of (B) Gβ3 (n=20-23, N=4) (C) Gβ1 (n=20-21, N=4) or (D) Kv7.4 (n=15, N=3) in renal artery myocytes in control morpholino or Gβ3 morpholino. 'n'=number of cells and 'N' = number of animals. Electrophysiology data was analysed by Bonferroni post-hoc test following a two-way ANOVA. IF data was analysed by one-way ANOVA with Bonferroni post hoc analysis. p<0.01 is denoted (**).

Figure 1.TIF

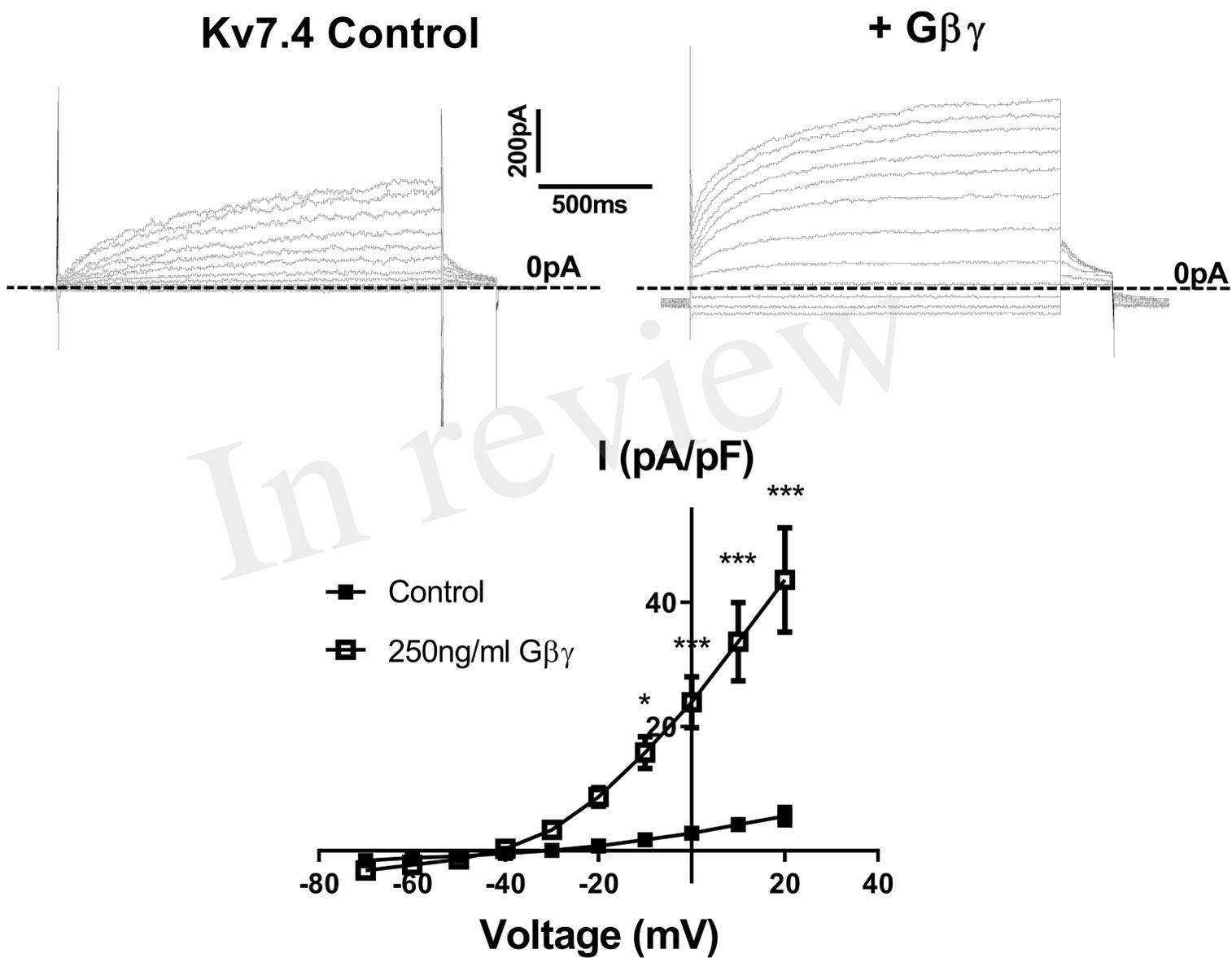


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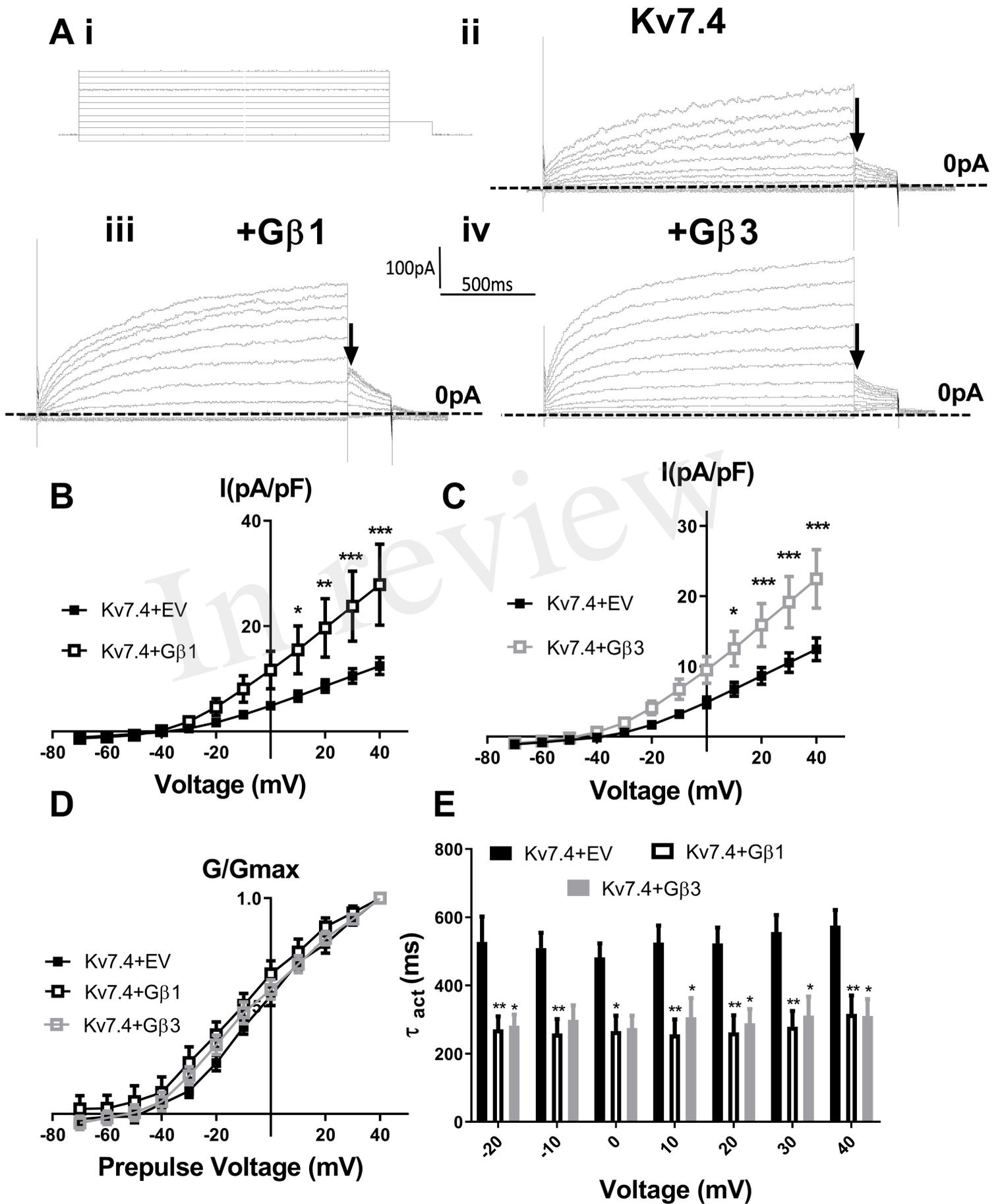


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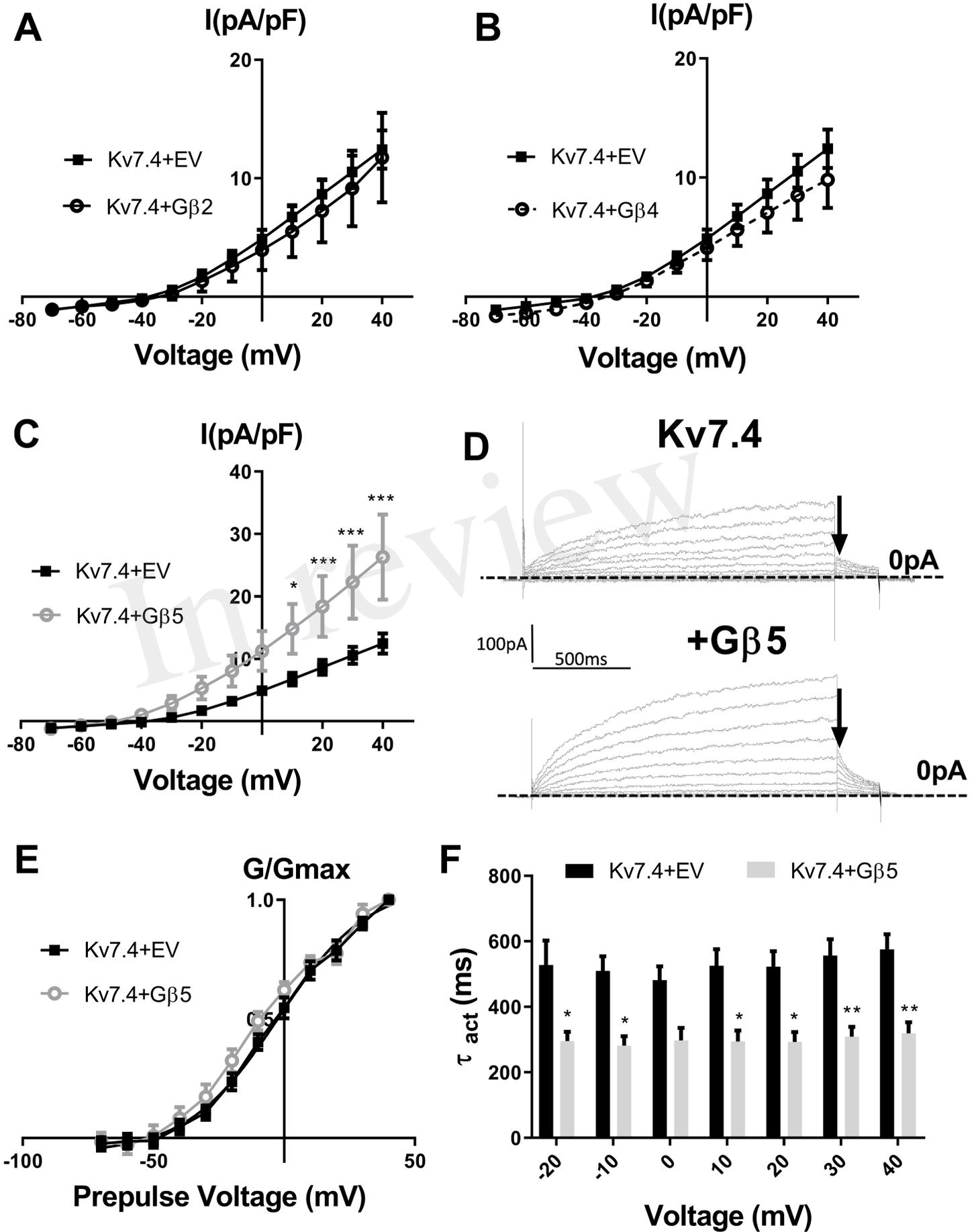


Figure 4.TIF

In review

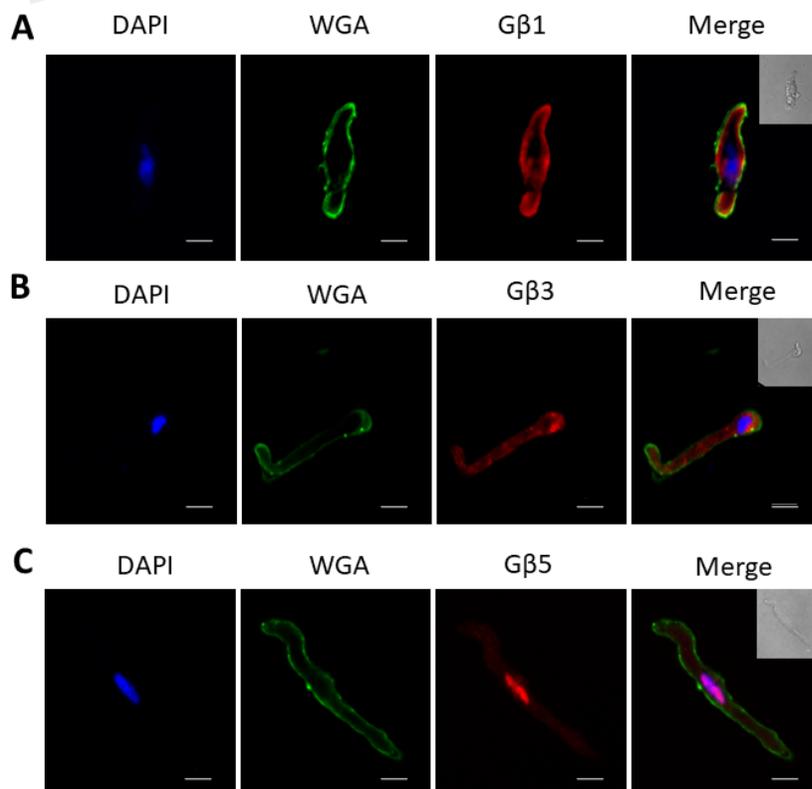


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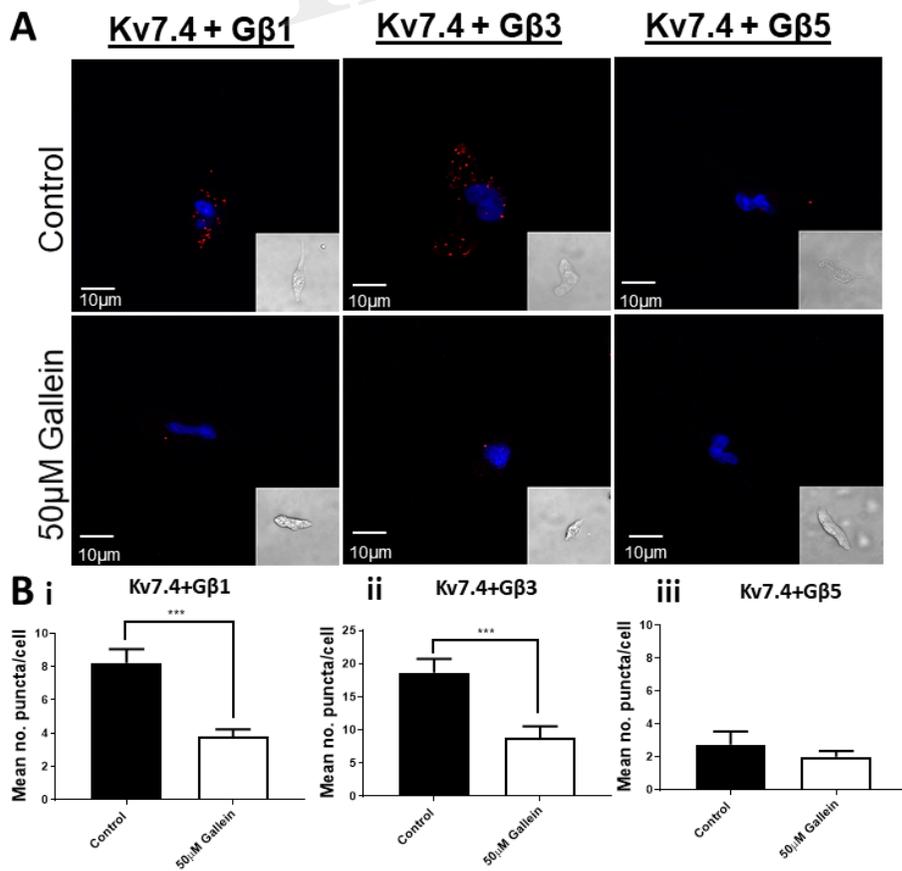


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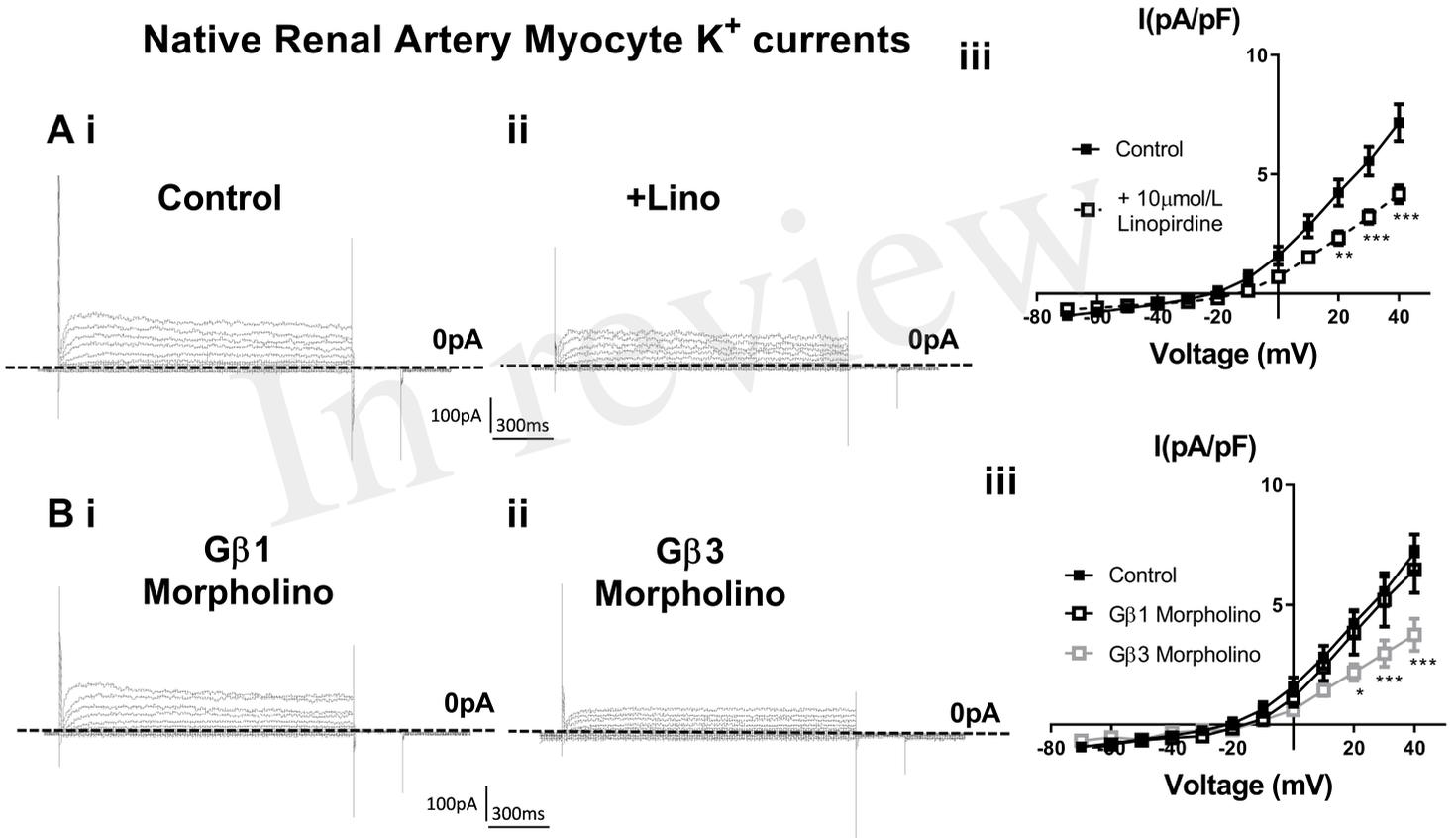


Figure 7.TIF

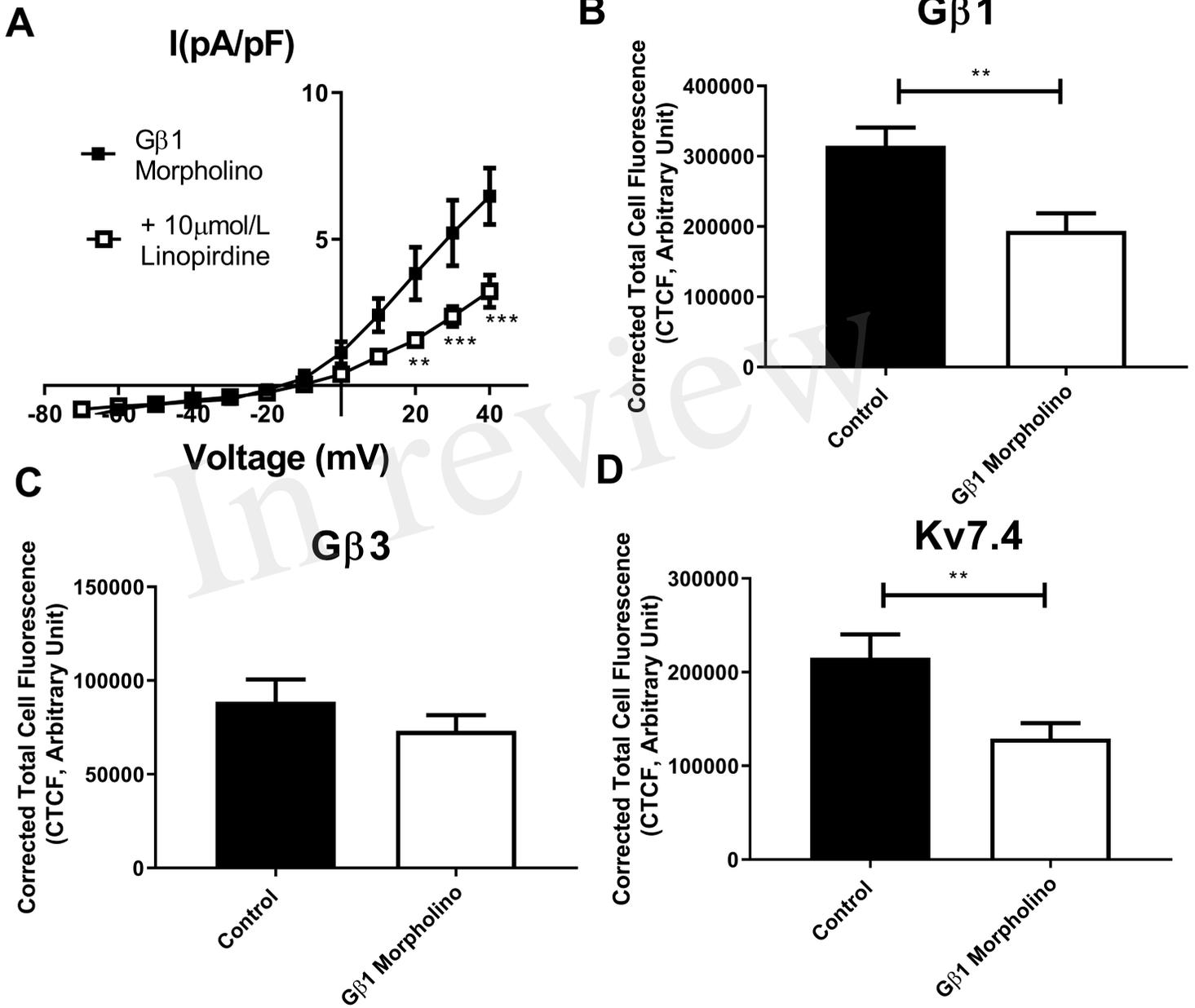


Figure 8.TIF

