G-protein βγ subunits are positive regulators of Kv7.4 and native vascular Kv7 channel activity

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Kv7.4 channels are a crucial determinant of arterial diameter both at rest and in response to endogenous vasodilators. However, nothing is known about the factors that ensure effective activity of these channels. We report that G-protein βγ subunits increase the amplitude and activation rate of whole-cell voltage-dependent K^+ currents sensitive to the Kv7 blocker linopirdine in HEK cells heterologously expressing Kv7.4, and in rat renal artery myocytes. In excised patch recordings, Gβγ subunits (2–250 ng /mL) enhanced the open probability of Kv7.4 channels without changing unitary conductance. Kv7 channel activity was also augmented by stimulation of G-protein–coupled receptors. Gallein, an inhibitor of Gβγ subunits, prevented these stimulatory effects. Moreover, gallein and two other structurally different Gβγ subunit inhibitors (GRK2i and a β-subunit antibody) abolished Kv7 channel currents in the absence of either Gβγ subunit enrichment or G-protein–coupled receptor stimulation. Proximity ligation assay revealed that Kv7.4 and Gβγ subunits colocalized in HEK cells and renal artery smooth muscle cells. Gallein disrupted this colocalization, contracted whole renal arteries to a similar degree as the Kv7 inhibitor linopirdine, and impaired isoproterenol-induced relaxations. Furthermore, mSIRK, which disassociates Gβγ subunits from α subunits without stimulating nucleotide exchange, relaxed precontracted arteries in a linopirdine-sensitive manner. These results reveal that Gβγ subunits are fundamental for Kv7.4 activation and crucial for vascular Kv7 channel activity, which has major consequences for the regulation of arterial tone.

Kv7 channels | KCNQ genes | G-protein beta gamma subunits | electrophysiology | vascular biology

Increased arterial constriction and lack of responsiveness to endogenous vasodilators is a hallmark of vascular disease leading to poor health prognosis. Defining the factors that determine vascular smooth muscle (VSM) activity and modulation by vasorelaxant molecules is therefore imperative for a better understanding of vascular disease. Potassium channels are key regulators of VSM tone because they promote membrane hyperpolarization that limits the activity of voltage-dependent calcium channels known to precipitate vasoconstriction (1). The Kv7 family of voltage-dependent potassium channels and the Kv7.4 isoform, in particular, has a fundamental role in this process. There are five Kv7 isoforms (Kv7.1–Kv7.5) of which Kv7.1, Kv7.4, and Kv7.5 are consistently expressed within VSM, where the predominant molecular architecture is a Kv7.4/Kv7.5 heterotetramer (2, 3). Activation of Kv7 channels produces relaxation of numerous arteries (4–8), whereas blockade of Kv7 channels results in contraction of vessels at rest (7, 9–11) or an inhibition of endogenously derived vasorelaxations (2, 11–13). In addition, molecular reduction of Kv7.4 reduces responses to various Gs-coupled vasodilators in a number of arteries (2, 11). Crucially, Kv7.4 abundance is reduced in various arteries from hypertensive animals (6, 11, 12) where relaxant responses to endogenous vasodilators are also impaired (11, 12). Despite the key role of Kv7.4 channels in the regulation of VSM, and their involvement in mediating Gs-coupled vasodilator responses, the factors that regulate channel activity are poorly understood, and the signals linking Kv7.4 to Gs-receptor activation remain to be elucidated.

G-protein–coupled receptor (GPCR) activation promotes the exchange of GDP for GTP resulting in disassociation of the heterotrimeric Gαβγ complex from the receptor into Gα-GTP and Gβγ (14). It is now established that the Gβγ complex as well as the Gα–GTP activates various intracellular signaling pathways (see refs. 15, 16 for reviews). Gβγ subunits also modulate various ion channels directly, a phenomenon of which there are only a handful of examples, with the positive regulation of an inwardly rectifying K^+ channel in the heart the best characterized (17, 18). In this study, we explored whether Gβγ subunits modulated Kv7.4 channels and therefore function as signaling intermediates following receptor stimulation. Our results show that not only are Gβγ subunits able to enhance Kv7 channels, but also that they are a crucial requirement for the basal activity of the Kv7.4 channel.

Results

Gβγ Subunit Enrichment Enhances Heterologously Expressed Kv7.4 Currents. Standard whole-cell patch-clamp experiments on a HEK293 cell line stably expressing Kv7.4 (19) were performed to ascertain the effect of Gβγ subunits. Membrane depolarization elicited robust K^+ currents that were stable after an initial equilibration period of 5 min and completely abolished by the Kv7 channel blocker linopirdine (10 μM) (Fig. S1 *B*, *i*). Intracellular perfusion of active Gβγ subunits (250 ng/mL) resulted in a steady increase in current amplitude that plateaued after 5–10 min (Fig. 1 *C* and *D*). Perfusion of boiled subunits had no significant effect on current amplitude compared with cells in which only standard

Significance

The Kv7.4 potassium channel is a critical regulator of vascular contractility both at rest and as a functional endpoint for a number of endogenous vasodilators. Despite its key role, nothing is known about the processes that determine Kv7.4 channel activity. We reveal an interaction between G-protein βγ subunits and Kv7.4 that is crucial for channel responses to membrane voltage. Blocking this interaction ablates channel activity, prevents β-adrenoceptor–mediated relaxation, and constricts renal arteries. Conversely, Gβγ subunits enhance Kv7.4 channels and produce arterial relaxation in a Kv7-dependent manner. This reveals a fundamental reliance of an ion channel on Gβγ subunits for basal activity, a previously unidentified finding, which has profound implications for vascular physiology and disease pathogenesis.

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The authors declare no conflict of interest.

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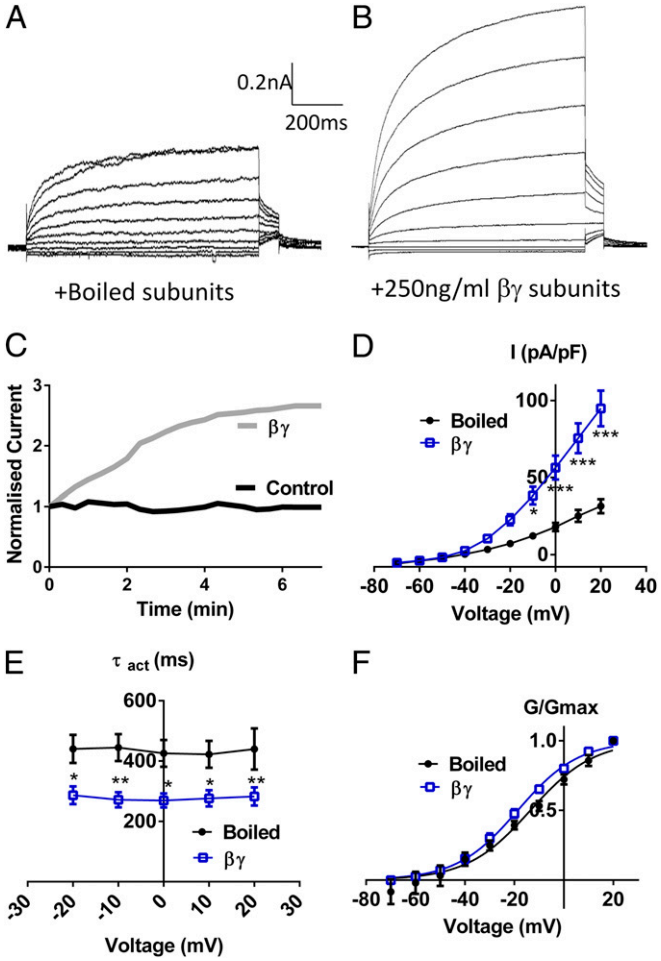

Fig. 1. Gβγ subunits enhance Kv7.4 channel activity. Representative voltage step recordings from HEK293 Kv7.4 cells perfused with 250 ng/mL Gβγ subunits either boiled (*A*) or active (*B*) and time course of current activation in both conditions (*C*). Mean IV relationships of boiled (n = 8) and active Gβγ (n = 12) (*D*). Analysis of rate of Kv7.4 current activation (*E*) and voltage dependence of activation (*F*) in the presence and absence of Gβγ subunits (n = 8–12). Error bars represent SEM, statistical analyses determined by two-way ANOVA where $P < 0.05$ is denoted (*), $P < 0.01$ is denoted (**), and $P < 0.001$ is denoted (***).

pipette solution was used (Fig. S1*A*) and Gβγ perfusion in untransfected HEK293 cells produced no increase in the negligible current present (Fig. S1, *B*, *ii*). The increase in Kv7.4 current amplitude by Gβγ subunit perfusion was associated with an increased rate of activation at membrane potentials of −20 mV upward (Fig. 1*E*) and a shift in the voltage dependence of activation (from −13.6 ± 1.5 to −18.9 ± 0.9 mV, n = 8–12, $P < 0.01$; Fig. 1*F*). The increase in current in the presence of Gβγ subunits was due to increased Kv7.4 activity and not a de novo recruitment of silent channels as currents recorded after intracellular dialysis with active or boiled Gβγ subunits were inhibited by linopirdine to the same level, and addition of Gβγ subunits had no effect in nontransfected HEK293 cells. *N*-ethylmaleimide (NEM) is a known stimulant of Kv7.4 (20) with biophysical effects similar to those produced by Gβγ. Application of 50 μM NEM increased Kv7.4 currents with an associated change in voltage dependence and activation kinetics, which was not seen in the presence of 250 ng/mL Gβγ, although τ_{act} was further increased by NEM (Fig. S2).

Inside-out excised patches from HEK293 Kv7.4 cells were performed to look at the effect of Gβγ subunits on Kv7.4 activity in the absence of intracellular mediators. Under basal conditions in patches voltage clamped at 0 mV, small amplitude currents with a relatively low open probability were recorded, similar to previous reports (20). The addition of Gβγ subunits (0.4–50 ng/mL) produced a concentration-dependent increase in apparent open probability (Fig. S3). Thus, with 2 ng/mL Gβγ subunits NPo increased from 0.035 ± 0.006 (n = 14) to 0.117 ± 0.034 (n = 3), $P < 0.05$ (Fig. S3); whereas after the addition of 50 ng/mL Gβγ subunits, it increased correspondingly to 0.343 ± 0.062 (n = 6), $P < 0.001$ (Fig. 2 *A*–*C*). Single-channel conductance was measured and calculated as 2.31 pS in agreement with ref. 20, and was not altered by addition of 50 ng/mL Gβγ (Fig. 2*D*).

Gβγ Subunit Inhibition Abolishes Kv7.4 Currents in HEK Cells. Having established that Gβγ subunits enhanced Kv7.4 channel activity, we investigated whether there was a constitutive effect of these mediators under control conditions; i.e., in the absence of any receptor stimulation or enrichment with Gβγ subunits. Consequently, we used three agents in whole-cell electrophysiology experiments that inhibit Gβγ activity in distinct ways; the small molecular inhibitor gallein (100 μM, in external solution; ref. 21), a peptide analog of the G-protein receptor kinase (Grk2i, 10μM, in pipette solution; ref. 22), and an antibody directed against the Gβ subunit (1:200, in pipette solution). Application of each of these agents resulted in complete ablation of the Kv7.4 currents evoked by membrane depolarization within 10 min (Fig. 3*A*). In cell-attached experiments application of gallein reduced markedly the open

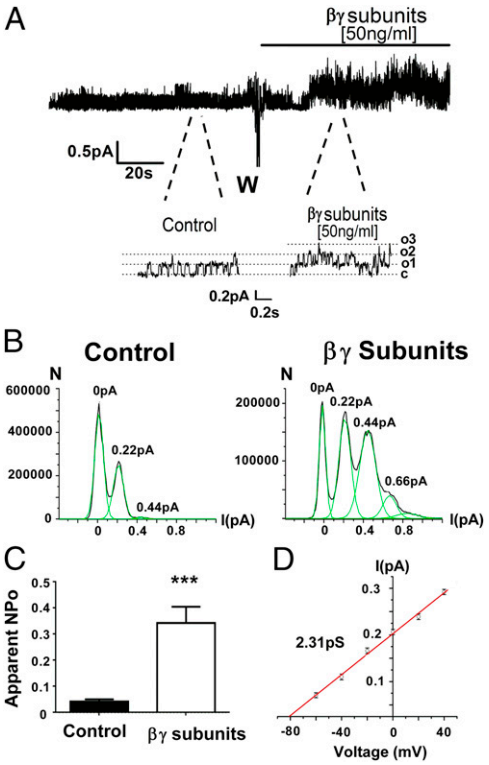

Fig. 2. Effect of Gβγ subunits on Kv7.4 single-channel activity. (*A*) Representative excised inside-out patch recordings of HEK293 Kv7.4 cells stimulated with Gβγ. Time course of recording in control and after addition of 50 ng/mL Gβγ subunits (solid bar) (*Upper*), with detailed view of channel openings (*Lower*). *W* denotes the artifact due to wash in of Gβγ subunits. (*B*) Amplitude histograms in control (*i*) and after addition of Gβγ subunits (*ii*), with Gaussian fit of channel open levels. (*C*) Mean apparent open probability in control (n = 14) and in the presence of 50 ng/mL Gβγ subunits (n = 6). (*D*) Current voltage relationship of excised patch Kv7.4 channels. Error bars represent SEM, statistical analyses determined by independent Student's t test, where $P < 0.001$ is denoted (***).

probability of Kv7.4 channels (Fig. 3*B*) without affecting channel conductance. These data reveal that Gβγ activity not only enhances Kv7.4 currents, but it is a necessary requirement for the basal activity of voltage-dependent Kv7.4 channels.

Gβγ Subunits Modulate Kv7-Dependent Currents in Renal Artery Myocytes. Physiologically, Kv7.4 channels are important modulators of VSM and have been linked to Gs-coupled vasodilatory responses in rat renal arteries (11). We recorded voltage-dependent currents in rat renal artery smooth muscle cells bathed in paxilline and 4-AP to remove extraneous K^+ currents and identified the Kv7 component as that sensitive to the Kv7-selective blocker linopirdine (10 μM). Intracellular perfusion of rat renal artery smooth muscle cells with 250 ng/mL Gβγ subunits produced a significant increase in the linopirdine-sensitive current (Fig. 4 *A* and *B*). This was associated with a concomitant increase in the rate of activation (Fig.4*C*). In perforated patch whole-cell recordings application of 100 μM gallein reduced the linopirdine-sensitive current significantly (Fig. 4*D*).

Receptor-Mediated Enhancement of Kv7 Channels. In a physiological setting, the level of free βγ subunits is increased by GPCR activation. We have previously shown a role for Kv7 channels in rat renal arteries in response to the β-adrenoreceptor agonist isoproterenol (11), so we undertook a series of experiments to ascertain whether this response involved βγ subunits. In rat renal myocytes patched with a mixture of K^+ channel blockers in the pipette (i.e., external) solution, small amplitude currents were recorded in cell-attached configurations that were not present if linopirdine (10 μM) was included in the pipette solution ($n = 4$). Isoproterenol (1 μM) applied to the bath increased the NPo of these channels from 0.025 ± 0.009 to 0.102 ± 0.023 ($n = 6$–7, $P < 0.01$; Fig. 5*A*). In the presence of gallein, basal NPo was negligible, and the stimulatory effect of isoproterenol was prevented (NPo 0.002 ± 0.001, $n = 5$). The presence of pertussis toxin (PTX) did not affect basal channel activity (0.026 ± 0.012, $n = 6$), and although isoproterenol induced an increase in channel activity (NPo 0.055 ± 0.033, $n = 5$), this was not statistically significant ($P = 0.45$). To further investigate receptor-mediated enhancement of Kv7.4, we used the purinergic receptors that are endogenous to HEK cells (P2Y only; ref. 23). As stimulation of P2Y receptors leads to activation of either pertussis-toxin–sensitive $G_i\alpha$ or PTX-resistant $G_q\alpha$, any enhancement of Kv7.4 currents would consolidate a role for liberated βγ subunits rather than the activated Gα-mediated signals. The P2Y ligand ATP (1 mM) increased the whole-cell Kv7.4 currents by 8.8 ± 2.7 pA/pF at 20 mV, ($P < 0.05$ compared with in control, $n = 8$; Fig. 5*B*) and significantly increased the rate of current activation (from 393 ± 52 ms in control to 216 ± 28 ms in 1 mM ATP, $P < 0.05$, $n = 8$). ATP produced no effect in the presence of either 100 μM gallein (change of 0.4 ± 0.5 pA/pF, $n = 6$) or 1 μg/mL PTX (change of −0.6 ± 1.9 pA/pF, $n = 6$; Fig. 5*B*). Overall, these studies reveal that liberation of βγ subunits by two different receptor mechanisms augments Kv7.4 channels and native Kv7 channel activity.

Kv7.4 and Gβ-Subunits Reside within the Same Nanodomain. Proximity ligation assay (PLA) allows the detection of proteins that reside within 40 nm of each other through fluorochrome generation by rolling circle amplification of proprietary oligonucleotides (24). We have previously used this technique to establish that the dominant molecular species of Kv7 channel in cerebral arteries is a Kv7.4/7.5 heteromer (2). In HEK293 cells stably expressing Kv7.4, an abundance of PLA punctae were recorded when Kv7.4 and pan-Gβ-subunit antibodies were used (Fig. 6*A*) consistent with the juxtaposition of these proteins.

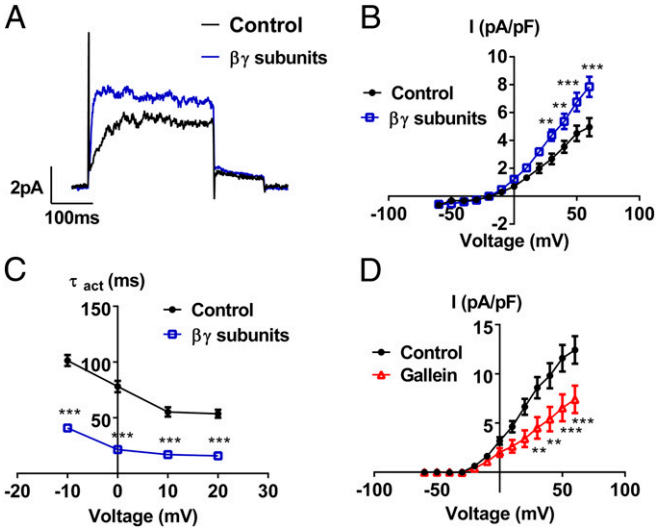

Fig. 4. Gβγ modulation of Kv7 channels in renal artery myocytes. Representative trace of voltage step recordings in rat renal artery myocytes in control ($n = 9$, black) and perfused with 250 ng/mL Gβγ subunits ($n = 9$, blue) (*A*), mean IV relationships depicting the linopirdine sensitive current (*B*) and analysis of rate of activation (*C*). Current voltage relationship of the linopirdine sensitive current in the presence and absence of 100 μM gallein (*D*) ($n = 7$). Error bars represent SEM, statistical analyses determined by two-way ANOVA where $P < 0.01$ is denoted (**) and $P < 0.001$ is denoted (***).

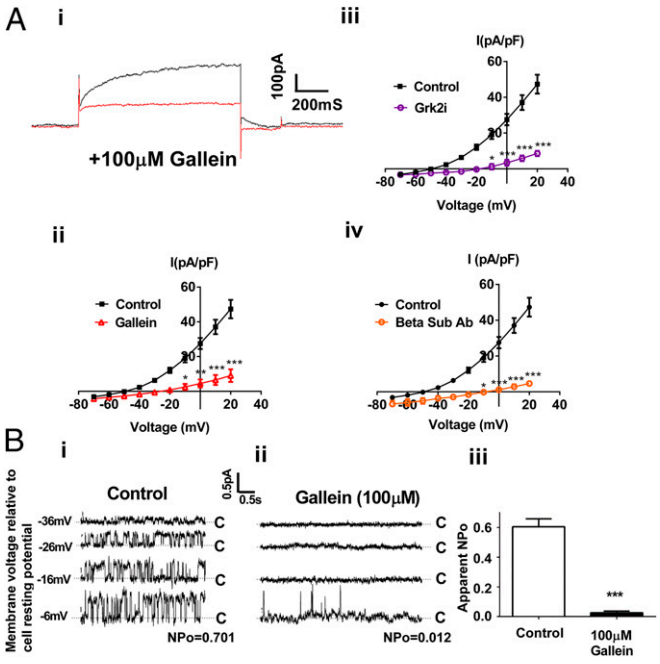

Fig. 3. Effect of Gβγ sequestration on Kv7.4 currents. (*A*) Representative current trace at +20 mV of control HEK293 currents in the absence (black) and presence of 100 μM gallein (red) (*i*), with mean IV relationships (*ii*). Mean IV relationships showing HEK293 Kv7.4 currents in the presence and absence of 10 μM GRK2i (*iii*) ($n = 7$) and a β-subunit antibody (*iv*) ($n = 6$). (*B*) Representative traces showing HEK293 Kv7.4 channel activity recorded in cell-attached configuration over a range of holding potentials (−20 to −50 mV). Therefore, relative to the resting membrane potential of HEK Kv7.4 cells (which is −56 mV; ref. 18), the expected voltage on the membrane is −36 to −6 mV, respectively. Channel activity can be seen in the absence (*B, i*) and in the presence of 100 μM gallein (*B, ii*) and mean data compared at −50 mV (*B, iii*) (control $n = 9$, gallein $n = 3$). Error bars represent SEM, statistical analyses determined by two-way ANOVA where $P < 0.05$ is denoted (*), $P < 0.01$ is denoted (**), and $P < 0.001$ is denoted (***).

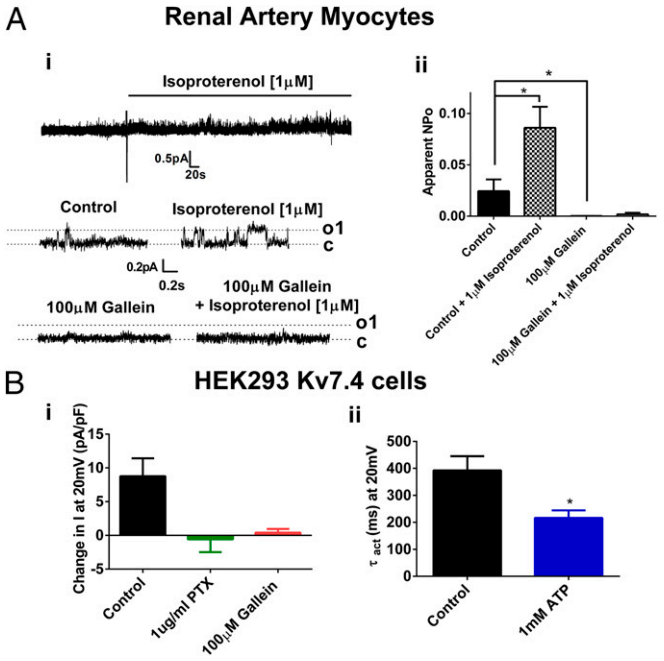

Fig. 5. Receptor-mediated activation of Kv7 channels. (*A, i*) Representative traces of a renal artery myocyte in cell-attached configuration before and after addition of 1 μM isoproterenol (solid bar). Time course of the experiment is shown in *Upper* and detailed channel openings can be seen in *Lower*. (*A, ii*) Mean NPo of renal artery myocytes in control or in 100 μM gallein before and after application of 1 μM isoproterenol (n = 5–7). (*B, i*) Mean change in current at 20 mV in HEK293 Kv7.4 cells after application of 1 mM ATP in control (n = 8) or in the presence of 1 μg/mL PTX (n = 6) or 100 μM gallein (n = 6). (*B, ii*) The mean rate of activation (τ_{act}) at 20 mV in HEK293 Kv7.4 cells in control and after application of 1 mM ATP (n = 8). Error bars represent SEM, statistical analyses determined by Student's t test where $P < 0.05$ is denoted (*).

The number of PLA punctae observed was reduced considerably by treatment of the cells with 100 μM gallein for 10 min (Fig. 6*B*), which is indicative of reduced interaction. A similar large number of PLA punctae were recorded in renal artery myocytes (Fig. 6*C*) that was diminished significantly after treatment with gallein (Fig. 6*D*). These data confirm a close interaction of Kv7.4 and Gβ subunits in renal artery smooth muscle cells at rest. However, in Western blot experiments no Gβ subunits were detected in the Kv7.4-immunoprecipitated fraction from Kv7.4 expressing HEK293 cells (Fig. S4) although Kv7.5 co-expressed in these HEKs was detected (Fig. S4).

Gβγ Subunits Are Involved in the Control of Vascular Tone. We assessed the functional role of Gβγ subunits on the activity of Kv7 channels on renal arteries at a whole-vessel level using isometric tension myography. In this artery, Kv7 blockade with linopirdine produces a robust contraction (Fig. 7*A*; also ref. 11). Application of 100 μM gallein contracted renal arteries to a level similar to that produced by linopirdine, which was not augmented further by subsequent application of the Kv7 blocker (Fig. 7 *A* and *B*) consistent with a role for Gβγ subunits dictating Kv7 activity. Also mSIRK (myristoylated-SIRKALNILGYPDYD), a cell-permeable Gβγ binding peptide, which causes disassociation of Gβγ subunits from α subunits without stimulating nucleotide exchange (25) relaxed precontracted renal arteries that were prevented by 10 μM linopirdine or 100 μM gallein (Fig. 7*C*). Isoproterenol relaxations of the rat renal artery are sensitive to Kv7 blockade or *KCNQ4* knockdown (ref. 11; Fig. 7 *D*, *i*). The addition of 100 μM gallein significantly inhibited isoproterenol-mediated relaxations of these vessels (Fig. 7, *D, ii*). These studies all consolidate the view that Gβγ subunit interaction is absolutely critical for effective function of vascular Kv7 channels and, when compromised, can lead to augmented vasospasm and diminished vasorelaxation.

Discussion

Our data has shown quite remarkably that Gβγ subunits are a crucial requirement for Kv7.4 channels to respond to membrane voltage. In HEK cells stably expressing Kv7.4, inhibition of Gβγ subunits by three distinct mechanisms completely abolished the voltage-dependent activity of the channel. This implies that Kv7.4 channels require active Gβγ subunits for their constitutive activity and that an association between Kv7.4 channels and Gβγ subunits exists under basal conditions. Indeed, our molecular PLA studies showed a colocalization of Kv7.4 and Gβ subunits in unstimulated HEK293 Kv7.4 cells, which was disrupted with gallein treatment. We also show that the amplitude of whole-cell currents produced by Kv7.4 overexpression was augmented by the addition of Gβγ subunits to the pipette solution, which was associated with an increased rate of current development and a leftward shift in the voltage dependence of activation. Similar effects on Kv7.4 current amplitude and kinetics were produced by ATP stimulation of endogenous P2Y receptors, which was prevented by pertussis toxin and the Gβγ subunit inhibitor gallein. Gβγ subunits are known to alter various signaling pathways (14, 15) but the stimulatory effects on Kv7.4 seem to not involve signal intermediates as they were also observed in inside-out excised patches recordings where Gβγ subunits enhanced the open probability without an effect on unitary conductance at concentrations as low as 2 ng /mL This sensitivity of Kv7.4 channels to Gβγ subunits is considerably greater than the well-characterized G-protein–gated inwardly rectifying K^+ channel (26).

To place our findings in more physiological context we examined the effects of Gβγ subunits in native renal artery smooth muscle cells where Kv7.4 is known to have a physiological role (11). However, in VSM, Kv7.4 channels do not exist in isolation, with Kv7.1 and Kv7.5 also present. In fact, the dominant

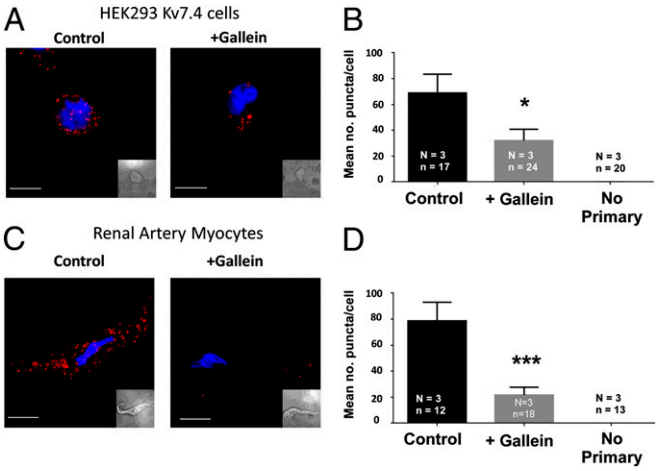

Fig. 6. In situ PLA detection of Kv7.4 and G_{β} subunit protein interactions in rat renal artery myocytes and KCNQ4 stably transfected HEK cells. Representative fluorescence and bright field (*Inset*) confocal midcell *xy* sections of (*A*) Kv7.4 stably transfected HEK293 cells or (*C*) renal artery myocytes, using primary antibodies for Kv7.4 and Gβ subunit together with appropriate PLA probes. Red puncta indicate target proteins are in close proximity (<40 nm). Nuclei are shown in blue as defined by DAPI. Quantification of the mean number of PLA signals per midcell *xy* section in (*B*) Kv7.4 stably transfected HEK cells or (*D*) renal artery myocytes. No primary controls produced no detectable PLA signal. The number of animals (*N*) and number of cells (*n*) for each treatment is shown. *$P < 0.05$, ***$P < 0.001$ for control versus gallein according to one-way ANOVA, multiple comparisons test. Scale bar represents 10 μm.

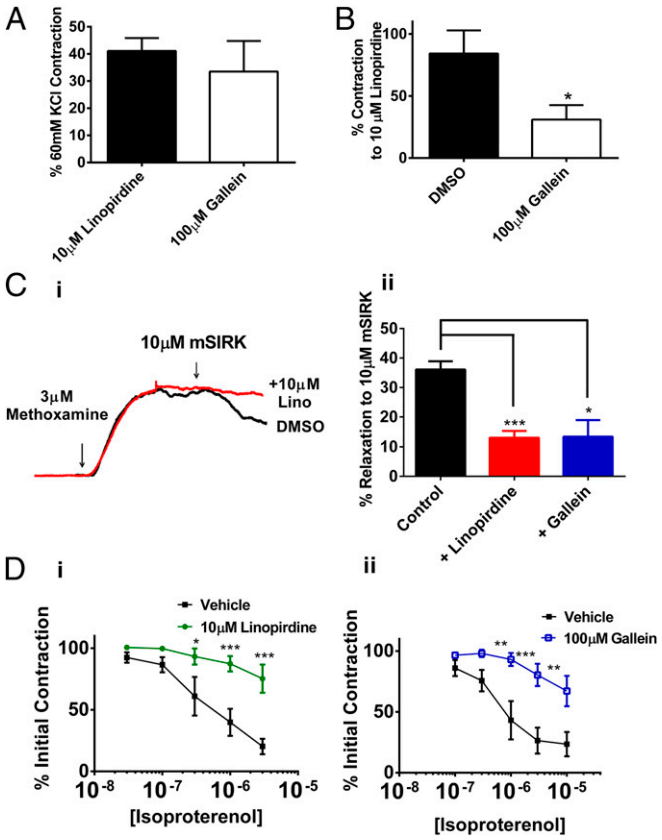

Fig. 7. Role of Gβγ subunits in vascular tone. (*A, i*) Mean contraction to 10 μM linopirdine or 100 μM gallein in renal arteries (relative to 60 mM KCl response, $n = 12$), and (*B*) mean contractions to 10 μM linopirdine in the presence or absence of 100 μM gallein ($n = 5$). (*C, i*) Representative trace of renal artery relaxation to 10 μM mSIRK in the absence and presence of 10 μM linopirdine and mean data (*C, ii*) ($n = 7$). (*D, i*) Concentration effect curves showing the mean percent relaxation of renal arteries (relative to the maximum 3 μM methoxamine-induced contraction) to increasing concentrations of isoproterenol in the absence and presence of 10 μM linopirdine ($n = 6$) and (*D, ii*) 100 μM gallein ($n = 6$). Error bars represent SEM, statistical analyses determined by two-way ANOVA where $P < 0.05$ is denoted (*), $P < 0.01$ is denoted (**), and $P < 0.001$ is denoted (***).

molecular architecture of vascular Kv7 channels in rat mesenteric and cerebral arteries is a heterotetramer formed from Kv7.4/Kv7.5 proteins in a 3:1 stoichiometry (2, 3). Bearing these caveats in mind, our PLA studies showed a colocalization of Kv7.4 and Gβ subunits in unstimulated rat renal artery myocytes, which was also disrupted with gallein treatment. Electrophysiological studies showed an opposing modulation of linopirdine-sensitive whole-cell currents by Gβγ and gallein although the reduction of Kv7 currents by gallein in native cells was less marked than in HEK cells expressing Kv7.4 cells. This may be the consequence of Kv7.4 /7.5 heteromers having a lower dependence upon Gβγ subunits than Kv7.4 homomers or the influence of interacting proteins such as auxiliary subunits not present in the HEK cell. Irrespective, these data show that native Kv7 channels in vascular myocytes are positively regulated by Gβγ subunits but are less reliant upon Gβγ subunits to function as the overexpressed Kv7.4 channels.

The role of Gβγ signaling in the regulation of VSM tone is not well characterized although this pathway contributes to vasorelaxations to calcitonin-gene–related peptide in the mesenteric artery (27). We found that isoproterenol-mediated relaxations, which are attenuated by Kv7 blockers and Kv7.4 knockdown (ref. 11), were sensitive to Gβγ blockade by gallein. In addition, mSIRK, which stimulates Gβγ dissociation without Gα activation, relaxed the rat renal artery in a linopirdine-sensitive manner. In support of a scheme where Gβγ subunits are required for the β-adrenoreceptor-induced augmentation of the vascular Kv7 channel we also showed that linopirdine-sensitive single-channel activity recorded in cell-attached configuration was enhanced by the β-adrenoreceptor agonist isoproterenol, which was prevented by gallein. This finding seems paradoxical as recording receptor-mediated effects with cell-attached configuration conventionally implies the involvement of a diffusible second messenger and Gβγ subunits are considered to be tethered to lipids in the cell membrane. One conclusion of our data are that Gα-mediated signals generated by β-adrenoreceptor activation require Gβγ interaction with the vascular Kv7 channel to alter activity, or that Gβγ subunits are involved in the postreceptor signaling. It is interesting to note that the cAMP analog, 8-bromo cAMP, increased Kv7.4 currents in HEK cells but not after the application of gallein (Fig. S5).

Gallein contracted renal arteries to the same extent as linopirdine; an effect which was not additive. These data imply that similar to the overexpressed Kv7.4 there is a tonic interaction of gallein-sensitive Gβγ subunits with vascular Kv7 channels to maintain relaxed arteries. At present we do not know the source of the Gβγ subunits for basal Kv7 channel activity. Some reports suggest that Gβγ subunits exist as a "free" pool close to the membrane (28), which is supported by the finding that Gα subunits have been located without Gβγ subunits (29). Alternatively Gβγ subunits may be liberated by a constitutively active receptor in close proximity to the channel. The inability to detect Gβ subunits in protein lysates from a Kv7.4 pull-down suggests the modulation may not be a simple direct interaction adds to the complexity. How Gβγ subunits enhance Kv7.4 activity or vascular Kv7 channels is an enigma yet to be unraveled and which could prove essential in our understanding of these channels.

Materials and Methods

Full experimental methods can be found in the *Supporting Information*.

Materials. G-protein βγ subunits from bovine brain and mSIRK were purchased from Merck. Gallein, Grk2i, and pertussis toxin were purchased from Tocris. All other chemicals were purchased from Sigma Aldrich. HEK293 cells stably expressing Kv7.4 were a gift from the University of Copenhagen (19).

Animals. Experiments were performed using male Wistar rats, between 12 and 15 wk of age (Charles River UK) culled by cervical dislocation in accordance with the UK Animal (Scientific Procedures) Act 1986.

Cell Isolation. Renal arteries were incubated at 37 °C in Ca^{2+}-free physiological salt solution (PSS) containing collagenase type IA (2 mg/mL) and protease type X (1 mg/mL) for 20 min followed by a 10-min wash in Ca^{2+}-free PSS at room temperature.

Electrophysiology. Experiments were conducted in whole-cell, cell-attached or excised-patch configurations (*Supporting Information*).

Proximity Ligation Assay. Rat renal artery myocytes were plated onto coverslips for 1 h. HEK293 Kv7.4 cells were seeded onto coverslips for 48 h. Both were then treated with gallein (100 μM) or vehicle control (DMSO) for 1 h. The Duolink in situ PLA detection kit 563 (Sigma) was used to visualize single molecule interactions for Kv7.4 and Gβ.

Coimmunoprecipitation. Immunoprecipitation of Kv7.4 was performed on protein lysates from HEK293 and HEK 293 Kv7.4 cells and probed for the presence of β-subunit using a conventional Western blot method.

Myography. Renal artery segments (~2 mm) were mounted in a myograph (Danish Myo Technology) for isometric tension recording (13). The chambers contained physiological salt solution, maintained at 37 °C and aerated with 95% O_2/5% CO_2. After normalization to 90% of the diameter at 100 mg Hg vessels were preconstricted with 3 μM methoxamine and responses to mSIRK (10 μM) or isoproterenol (30 nM–10 μM) were determined in control (DMSO) and in the presence of linopirdine (10 μM) or gallein (100 μM).

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