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Fast inactivation of a brain K^+ channel composed of $K_v1.1$ and $K_v\beta1.1$ subunits modulated by G protein $\beta\gamma$ subunits

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Modulation of A-type voltage-gated K^+ channels can produce plastic changes in neuronal signaling. It was shown that the delayed-rectifier $K_v1.1$ channel can be converted to A-type upon association with $K_v\beta1.1$ subunits; the conversion is only partial and is modulated by phosphorylation and microfilaments. Here we show that, in *Xenopus* oocytes, expression of $G\beta_1\gamma_2$ subunits concomitantly with the channel (composed of $K_v1.1$ and $K_v\beta1.1$ subunits), but not after the channel's expression in the plasma membrane, increases the extent of conversion to A-type. Conversely, scavenging endogenous $G\beta\gamma$ by co-expression of the C-terminal fragment of the β-adrenergic receptor kinase reduces the extent of conversion to A-type. The effect of $G\beta\gamma$ co-expression is occluded by treatment with dihydrocytochalasin B, a microfilament-disrupting agent shown previously by us to enhance the extent of conversion to A-type, and by overexpression of $K_v\beta1.1$. $G\beta_1\gamma_2$ subunits interact directly with GST fusion fragments of $K_v1.1$ and $K_v\beta1.1$. Co-expression of $G\beta_1\gamma_2$ causes co-immunoprecipitation with $K_v1.1$ of more $K_v\beta1.1$ subunits. Thus, we suggest that $G\beta_1\gamma_2$ directly affects the interaction between $K_v1.1$ and $K_v\beta1.1$ during channel assembly which, in turn, disrupts the ability of the channel to interact with microfilaments, resulting in an increased extent of A-type conversion.

Keywords: channel assembly/fast inactivation/Gβγ/potassium channel

Introduction

Voltage-gated K^+ (K_v) channels are involved in a host of cellular processes from setting the resting membrane potential and shaping action potential wave-form and frequency, to controlling synaptic strength (Rudy *et al*., 1988). Two major types of K_v channels have been described. The delayed-rectifier type inactivate slowly with a time course of up to several seconds (C-type inactivation) and are involved in repolarization of action potential and attenuation of cell excitability. The A-type channels inactivate rapidly with a time course of less than ~100 ms (N-type inactivation) and are involved in regulation of firing patterns and the threshold for firing, and thus play a key role in encoding pre- and postsynaptic nervous signals (Connor and Stevens, 1970). It has been shown that delayed-rectifier channels can be converted to A-type channels upon association of pore-forming *Shaker*-related (K_v1; Stuhmer *et al*., 1989) α subunits with peripheral auxiliary β subunits ($K_v\beta1$; Rettig *et al*., 1994). The rapid inactivation results from occlusion of the inner mouth of the channel pore with the N-terminal part of the $K_v\beta1$ subunit (Rettig *et al*., 1994), in a mechanism termed 'ball-and-chain' inactivation (Armstrong and Bezanilla, 1977). However, we showed, by studying the hetero-multimeric channels composed of $K_v1.1$ and $K_v\beta1.1$ subunits, that conversion of the delayed-rectifier to A-type is not complete and is subject to modulations by cellular processes. The extent of conversion to A-type is dependent on interactions of the channel with microfilaments and on cellular mechanisms leading to phosphorylation (Levin *et al*., 1996) and dephosphorylation (Levy *et al*., 1998) of Ser446 at the C-terminus of $K_v1.1$. Part of the effect of phosphorylation is conveyed via regulation of the interaction of the $K_v1.1$'s C-terminus with a postsynaptic density-95 (PSD-95)-like protein (Jing *et al*., 1997). In this study, we identify G protein βγ subunits as a cellular regulator of the channel's interaction with microfilaments and consequently as a regulator of A-type conversion. Such modulations of A-type activity that modify K^+ channel gating properties can produce plastic changes in neuronal signaling and may be involved in neural mechanisms related to behavior and learning (Crow, 1988).

The large GTP-binding proteins (G proteins), made up of $G\alpha\beta\gamma$ heterotrimers, dissociate into Gα and Gβγ subunits, which interact separately with effector molecules. While Gα has been recognized for many years now as a signal-transducing molecule, Gβγ has been recognized only recently as a direct regulator of many target proteins (Clapham and Neer, 1997). Many ion channels are modulated by G proteins, most by activating intracellular cascades and some via direct interaction with the channel proteins (Wickman and Clapham, 1995). Gβγ has been demonstrated to inhibit some voltage-dependent Ca^{2+} channels via direct interactions (for a review see Dolphin, 1997) and to activate the G protein-activated K^+ channels (GIRKs) by direct binding (for a review see Dascal, 1997). Very recently, it has been demonstrated that co-expression of Gβγ with rat brain type IIA voltage-gated Na^+ channels induces a persistent current component with shifted voltage dependence of inactivation. Direct Gβγ interaction with the channel has been inferred in this case from results showing that peptides containing Gβγ-binding motifs from adenylyl cyclase 2 and the Na^+ channel inhibited the Gβγ

effect. Our present report is the first demonstration of a modulation of a voltage-gated K^+ channel by direct interaction with Gβγ. Also, our results point to a novel mechanism of action by Gβγ.

Immunocytochemical studies have shown that $K_v1.1$ and $K_v\beta1.1$ proteins are widely distributed in adult rodent brain and occur in specific neuronal locations, in particular in juxtaparanodal regions of myelinated axons and in terminal fields (Sheng *et al*., 1993; Wang *et al*., 1993, 1994; Rhodes *et al*., 1995, 1996). This may indicate a role in axonal action potential propagation and repolarization of the membrane at the synaptic terminal at specific brain regions, e.g. cerebellar Purkinje cells where high immunoreactivity of both proteins have been detected (Veh *et al*., 1995; Rhodes *et al*., 1996). Modulation of A-type activity by mechanisms described in our previous publications and in this report may play a significant role in the regulation of neuronal excitability in these regions.

Results

Level of expression of G protein βγ subunits (Gβγ) affects the extent of inactivation of the $K_v1.1$–$K_v\beta1.1$ current

When $K_v1.1$ (α) is co-expressed with $K_v\beta1.1$ (β) in oocytes injected with the corresponding mRNAs, a heteromultimeric $K_v1.1$–$K_v\beta1.1$ channel is formed that conveys a rapidly inactivating current with a fast inactivating component (*I*i) and a non-inactivating sustained component (*I*s). *I*s is defined as the current remaining at the end of a 120 ms depolarizing pulse to +50 mV (Levin *et al*., 1996; see graphs in Figure 1A). The extent of inactivation (the inactivating fraction) is defined as *I*i/*I*p (*I*p = peak current) (Levin *et al*., 1996). Previously (Levin *et al*., 1996), we showed that the extent of inactivation of the $K_v1.1$–$K_v\beta1.1$ current, and not the inactivation rate constant, increases up to saturation at *I*i/*I*p = 0.5–0.8 (depending on the batch of oocytes) as the injected mRNA ratio of $K_v\beta1.1$ to $K_v1.1$ (β:α) is increased up to a ratio of ~50:1 (depending on the batches of mRNAs and oocytes). In parallel, the amount of $K_v\beta1.1$ protein co-precipitated with $K_v1.1$ protein, i.e. the number of $K_v1.1$ subunits that associate with $K_v\beta1.1$ subunits, increases until saturation is reached at a β:α mRNA ratio of ~50:1.

To examine the effect of G protein βγ subunits on the inactivation of the $K_v1.1$–$K_v\beta1.1$ current, we co-expressed $G\beta_1\gamma_2$ subunits with the channel subunits and recorded currents in a two-electrode voltage clamp configuration (Figure 1; normalized values are used when average values are derived from several batches of oocytes because of variability among batches). In oocytes expressing the channel subunits at saturating ratios, Gβγ co-expression occasionally increased the extent of inactivation of the current that was assayed 2 days after mRNA injection. However, when averaged over many experiments, the effect was very small (Figure 1B). Reducing the β:α ratio below saturation (between 30:1 and 3:1) resulted in a marked and reproducible increase in the extent of inactivation in oocytes expressing Gβγ subunits (Figure 1A and B). A test performed in a single batch of oocytes showed that the lower the β:α ratio the larger the degree of increase in extent of inactivation (Figure 1C, left panel). Actually, at the saturating β:α ratio (50:1), the extent of inactivation of the channel was so high that Gβγ could not increase it further. The inactivation time constant (τ_{inact}) for channels in the presence and in the absence of Gβγ did not differ statistically and were determined in two batches of oocytes to be 4.96 ± 0.25 ms (n = 22) and 5.04 ± 0.09 ms (n = 25), respectively. Also, Gβγ expression did not affect the voltage dependence of activation of the channel (Figure 1A, inset). Gβγ co-expression with $K_v1.1$ subunits alone had no effect on the kinetics of the slowly inactivating (C-type inactivation) $K_v1.1$ current (not shown). Conversely, in oocytes co-expressing the C-terminal region of β-adrenergic receptor kinase (C-βARK1; in-frame with the first 15 N-terminal amino acids of src60), the extent of inactivation was reduced in 12 out of 18 batches of oocytes tested (Figure 1A and B). This fragment of C-βARK was shown previously to bind Gβγ subunits effectively (Koch *et al*., 1993) and to inhibit the activation of the G protein-coupled potassium channel (GIRK) in *Xenopus* oocytes (E.Reuveny, unpublished data). The presence of the N-terminal src60 amino acids was used to target the C-βARK1 fragment to the membrane (Dascal *et al*., 1995). The C-βARK effect was about the same for saturating and non-saturating β:α ratios (Figure 1C, right panel), suggesting a constitutive role for Gβγ in the inactivation and the existence of sufficient free endogenous Gβγ to sustain maximal inactivation attained at the saturating β:α ratio.

In oocytes expressing C-βARK, the current amplitudes were ~50% of those in oocytes not expressing C-βARK; the reason for this is not known.

Interrelationships between the Gβγ effect, channel phosphorylation and microfilaments

Previously we showed that the extent of inactivation of the $K_v1.1$–$K_v\beta1.1$ channel can be increased by treatment of oocytes with dihydrocytochalasin B (DHCB), leading to microfilament depolymerization (Levin *et al*., 1996), presumably due to disruption of points of interaction between the channel and microfilaments. One point of interaction was found to be the very end of the C-terminus of the $K_v1.1$ subunit via a PSD-95-like protein (Jing *et al*., 1997). We also showed that phosphorylation of $K_v1.1$ at Ser446 is another factor that affects the extent of inactivation. The extent of inactivation of the wild-type channel which is substantially phosphorylated is larger than that of the unphosphorylated $K_v1.1_{S446A}$–$K_v\beta1.1$ channel (Levin *et al*., 1996); part of the effect of phosphorylation is due to its modulation of the C-terminal interaction of $K_v1.1$ with microfilaments (Jing *et al*., 1997). In this study, we set out to test the relationship of these inactivation-affecting factors to the Gβγ effect. To examine the relationship of the Gβγ effect to phosphorylation, we examined oocytes co-expressing Gβγ with the $K_v1.1_{S446A}$–$K_v\beta1.1$ channel and found that the increase in inactivation (Figure 2A, left panel) was similar to that of the wild-type channel (compare with Figure 1B). To test the relationship with the C-terminal-mediated interaction with microfilaments, Gβγ was co-expressed with channels of which the last amino acids of $K_v1.1$ that interact with the PSD-95-like proteins (Kim *et al*., 1995) were deleted (K490s.c; Jing *et al*., 1997). An experiment performed on

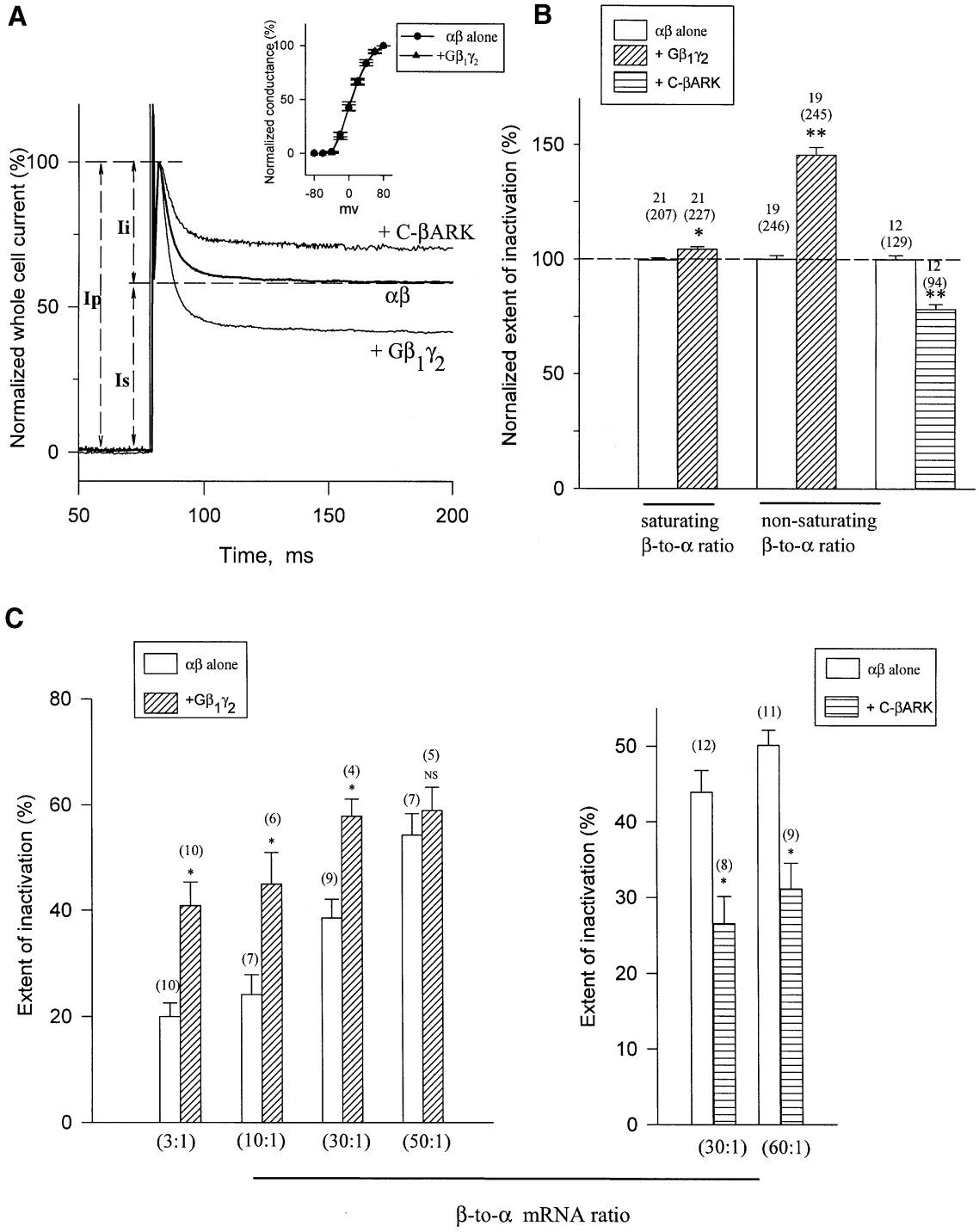
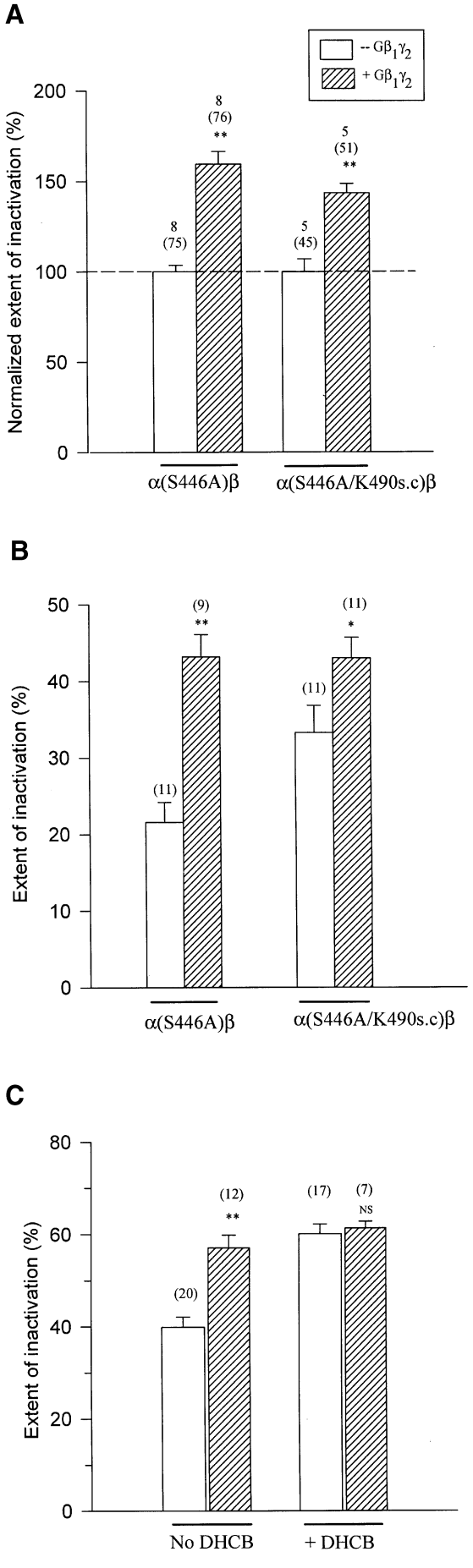


Fig. 1. Co-expression of Gβγ or C-βARK with $K_v1.1$ (α) and $K_vβ1.1$ (β) affects the extent of inactivation of the K^+ currents in *Xenopus* oocytes. (**A**) Normalized current traces evoked by a depolarization to +50 mV recorded by the two-electrode voltage clamp technique from single oocytes injected with either $K_vβ1.1$ and $K_v1.1$ mRNAs alone at a $K_vβ1.1$:$K_v1.1$ (β:α) ratio of 30:1 or co-injected with either $Gβ_1γ_2$ or C-βARK mRNAs; $τ_{inact}$s were 4.98, 4.61 and 5.30, respectively. *Ii*, *Is* and *Ip* illustrate definitions in the text for the inactivating, non-inactivating and total current components of $K_v1.1$–$K_v1.1$ (αβ), respectively. The inset shows activation curves of currents elicited in oocytes injected with $K_v1.1$ and $K_vβ1.1$ alone or co-injected with $Gβ_1γ_2$ (four oocytes each) by 250 ms depolarizations to the indicated voltages, at 20 mV increments, every 20 s (to allow recovery from inactivation); the elicited currents were normalized to maximal current at +80 mV. (**B**) Normalized and averaged effects of $Gβ_1γ_2$ mRNA co-injection with saturating β:α mRNA ratios (50:1 to 200:1; left panel) and with non-saturating ratios (3:1 to 30:1; middle panel), and effect of C-βARK mRNA co-injection (right panel). $*p < 0.004$; $**p < 0.0000001$. Numbers above the bars indicate the number of batches of oocytes; numbers in parentheses indicate the number of oocytes. (**C**) Effect of $Gβ_1γ_2$ (left panel) or C-βARK (right panel) mRNAs co-injection with different β:α mRNA ratios, each assayed in a single batch of oocytes; shown are absolute values of inactivations. $*p < 0.02$; NS, not significant.

a single batch of oocytes (Figure 2B) showed that this truncation increased the extent of inactivation, as shown before (Jing *et al*., 1997), and Gβγ co-expression further increased it but only up to about the same level as in the untruncated channel co-expressed with Gβγ. Figure 2A (right panel) shows normalized and averaged results of



several such experiments. Thus, the Gβγ effect occluded the effect of disruption of the C-terminal interaction with microfilaments. To test the relationship between the Gβγ effect and the overall interaction of the channel with microfilaments, the effect of a 4 h treatment of oocytes with DHCB on channels co-expressed with Gβγ was examined and compared with that in the absence of Gβγ in two batches of oocytes (Figure 2C). DHCB treatment increased the inactivation up to about the same level as Gβγ expression. In oocytes expressing Gβγ, the DHCB treatment had a small effect, if any.

Gβ interacts physically with the channel

To probe for possible physical interaction between G protein βγ subunits and the channel $K_v1.1$ and $K_v\beta1.1$ subunits, we have measured the *in vitro* binding of ^{35}S-labeled $G\beta_1\gamma_2$ synthesized in reticulocyte lysate to GST fusion proteins corresponding to the major intracellular parts of $K_v1.1$ and a part of $K_v\beta1.1$ subunits. The following GST fusion proteins were used: GSTαC, corresponding to amino acids 412–495, i.e. the whole C-terminus of $K_v1.1$; GSTαN1 and GSTαN2, corresponding to the whole of (amino acids 1–167) and most of the N-terminus (amino acids 14–162) of α, respectively; GSTαT1'B' corresponding to amino acids 72–143 of the N-terminus region that is involved in tetramerization of $K_v\alpha$ subunits (Li *et al*., 1992; Shen *et al*., 1993; Shen and Pfaffinger, 1995) and in $K_v\beta$ subunit binding (includes some residues essential for $K_v\beta$ binding; Sewing *et al*., 1996; Yu *et al*., 1996); and GSTβ_V, corresponding to amino acids 1–72 of $K_v\beta1.1$, i.e. a domain that is variable among the different $K_v\beta$ proteins and is not involved in binding to $K_v\alpha$. The scheme shown in Figure 3A illustrates the position of the different parts of $K_v1.1$. Figure 3B shows that Gβ bound to αN1, αN2 and β_V but not to αC. Since ^{35}S-labeled $G\beta_1$, and not $G\gamma_2$, each synthesized alone in reticulocyte lysate, bound to the above proteins (not shown), we concluded that the $G\beta_1$ interacts physically with the channel. Next, we tested the relationship between the binding of Gβ and $K_v\beta1.1$ (^{35}S-labeled and synthesized in reticulocyte lysate). Figure 3C shows that $K_v\beta1.1$ bound to αN1 and αN2, as expected; its binding was more pronounced than that of Gβ. However, while Gβ bound to αNT1'B', $K_v\beta1.1$ did not. In the presence of Gβγ, $K_v\beta1.1$ binding to αN1 was reduced by ~30% (not shown); this was probably not due to specific competitive binding of the two proteins to the N-terminus of $K_v1.1$, but rather to non-specific limitations of the experimental method, as $K_v\beta1.1$ binding to αN1 was reduced to the same extent

Fig. 2. Relationships of the Gβγ effect to the channel's phosphorylation and interactions with microfilaments. (**A** and **B**) Effects of $G\beta_1\gamma_2$ mRNA co-injection with $K_v\beta1.1$ (β) and either $K_v1.1_{S446A}$ [α (S446A)] or $K_v1.1_{S446A/K490s.c}$ [α (S446A/K490s.c.)] mRNAs in non-saturating β:α mRNA ratios. The Gβγ effect was normalized and averaged over several batches of oocytes (A) or shown in a single batch injected with a β:α mRNA ratio of 7:1 (B). Numbers above the bars indicate the number of batches of oocytes; numbers in parentheses indicate the number of oocytes. (**C**) Effect of $G\beta_1\gamma_2$ mRNA co-injection with $K_v1.1$ (α) and $K_v\beta1.1$ (β) mRNAs in non-saturating ratios in oocytes of a single batch that were not treated or treated with DHCB. $^{**}p < 0.0004$; $^{*}p < 0.04$; NS, not significant.

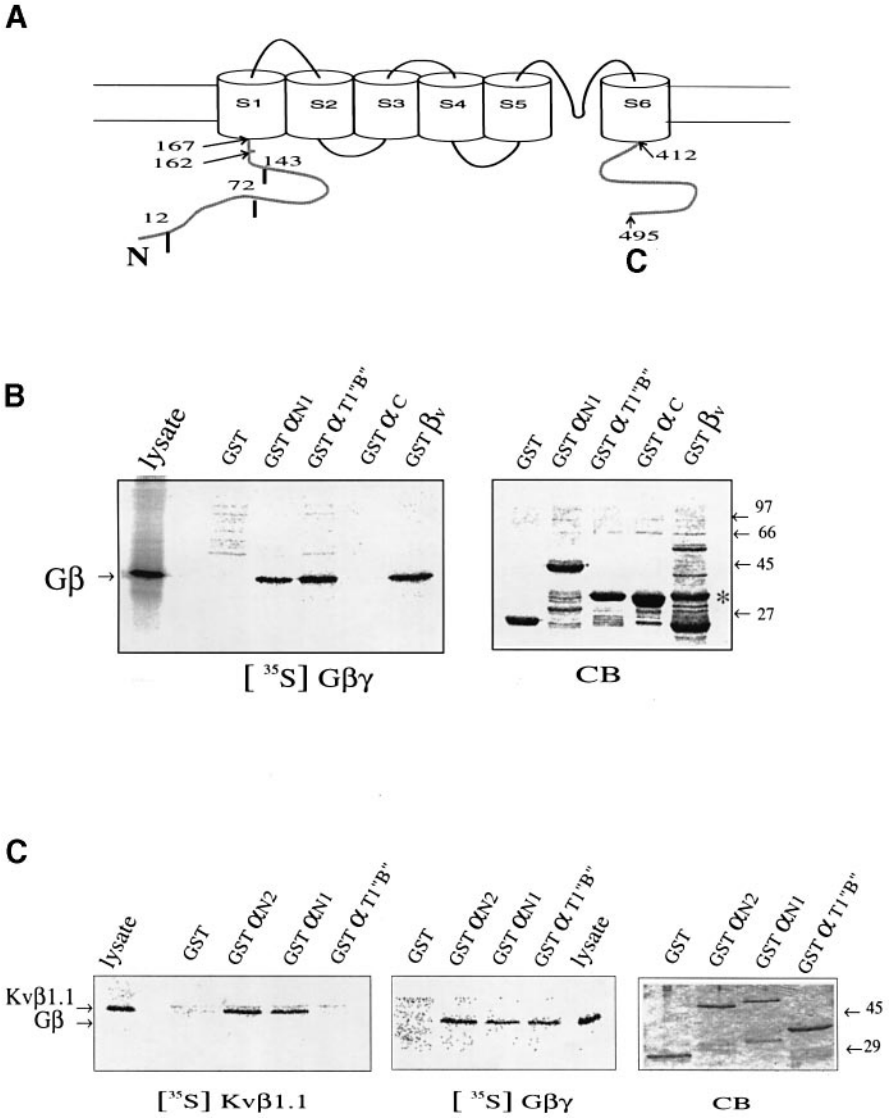


Fig. 3. Interaction of *in vitro* synthesized ^{35}S-labeled $G\beta_1\gamma_2$ and $K_v\beta1.1$ with GST fusion proteins of $K_v1.1$ and $K_v\beta1.1$ fragments. (**A**) Schematic presentation of the $K_v1.1$ subunit. (**B**) Left panel: interaction of Gβγ with the GST fusion proteins corresponding to parts of $K_v1.1$ and $K_v\beta1.1$. Right panel: Coomassie Blue staining of the same gel. (**C**) Left and middle panels: comparison of the interaction of $K_v\beta1.1$ and $G\beta_1\gamma_2$ with GST fusion proteins of $K_v1.1$ in a single experiment. Right panel: Coomassie Blue staining of one of the two gels that were run in parallel. The proteins were separated by SDS–12% PAGE and monitored using a PhosphorImager. αN1, αN2 and αT1'B' correspond to whole (amino acids 1–167), most of the length (amino acids 14–162) and tetramerization domain 'B' (amino acids 72–143) of the $K_v1.1$ N-terminus, respectively; αC corresponds to the whole C-terminus of $K_v1.1$ (amino acids 412–495); β_V corresponds to the variable region of $K_v\beta1.1$ (amino acids 1–72). Similar results to those shown in (B) and in (C) were obtained in four and two additional experiments, respectively. * Indicates the predicted position of the fusion protein. Numbers on the right refer to the mobility of pre-stained molecular weight standards.

in the presence of PSD-95 protein synthesized in the lysate that binds to αC but not to αN1 (not shown).

Co-expression of Gβγ increases the capacity of $K_v1.1$ to bind $K_v\beta1.1$ subunits

Next, we addressed the question of whether Gβγ co-expression affects the interaction between the channel $K_v1.1$ and $K_v\beta1.1$ subunits. We performed two kinds of biochemical analyses of the channel proteins. In the first kind of experiment, Western blot analysis of $K_v\beta1.1$ protein was done in oocytes tested electrophysiologically on the same day and displayed a pronounced Gβγ effect. For this purpose, we co-immunoprecipitated the channel subunits by antibody directed against $K_v1.1$, and they were subjected to SDS–PAGE, blotted onto membranes and probed with antibody directed against $K_v\beta1.1$ subunit for co-precipitated $K_v\beta1.1$ protein. In parallel, proteins of oocytes from the same batch were subjected directly to SDS–PAGE analysis without immunopurification, blotted and probed for total-$K_v\beta1.1$ protein. The amount of $K_v\beta1.1$ protein co-precipitated with $K_v1.1$ was quantified and normalized for total $K_v\beta1.1$ protein. In four such experiments (see a representative experiment in Figure 4A), the normalized co-precipitated $K_v\beta1.1$ protein was 2.0 ± 0.15 ($p < 0.001$) fold larger in oocytes expressing Gβγ than in oocytes that did not express Gβγ, suggesting that co-expression of Gβγ increases the capacity of $K_v1.1$ subunits to bind $K_v\beta1.1$ subunits in correlation with the increase in inactivation.

A second type of biochemical analysis was done in an

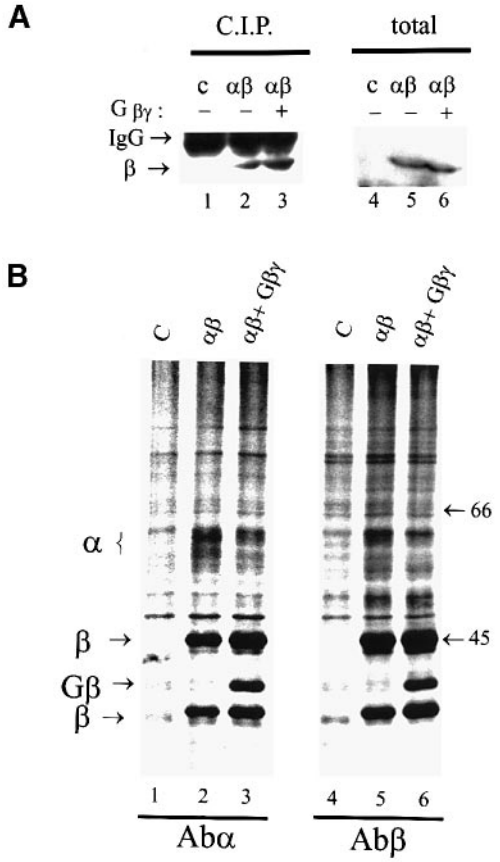


Fig. 4. Digitized PhosphorImager scans of SDS–PAGE analyses of the effect of Gβγ on the association of $K_v\beta1.1$ (β) with $K_v1.1$ (α) proteins. (**A**) Immunoblot analysis of $K_v\beta1.1$ in oocytes previously tested electrophysiologically. Proteins were precipitated from homogenates of oocytes either uninjected (c; lanes 1 and 4), injected with $K_v1.1$ and $K_v\beta1.1$ mRNAs alone (lanes 2 and 5) or co-injected with $G\beta_1\gamma_2$ (lanes 3 and 6), with either an antibody against $K_v1.1$ followed by protein A–Sepharose (left panel) or with ethanol (right panel), electrophoresed on an 8% gel, transferred to PVDF transfer membranes and the resultant immunoblots probed with anti-$K_v\beta1.1$ antibody to monitor the amounts of co-immunoprecipitated (C.I.P.; left panel) or total (right panel) $K_v\beta1.1$. Signals were visualized using ECL. (**B**) Analysis of [^{35}S]Met/Cys-labeled $K_v\beta1.1$ and $K_v1.1$ proteins co-immunoprecipitated by either $K_v1.1$ (left panel) or $K_v\beta1.1$ antibodies (right panel) from homogenates of oocytes injected with the same combinations as in (A) but with 10-fold larger $K_v1.1$ and $K_v\beta1.1$ mRNA concentrations. The arrow indicating Gβ points to Gβ that was pulled down by protein A–Sepharose (see text). Numbers on the right refer to the mobility of pre-stained molecular weight standards.

effort to resolve by SDS–PAGE, in addition to $K_v\beta1.1$, also the $K_v1.1$ protein. Both proteins were expressed from mRNA concentrations that were ~10-fold larger than those in the electrophysiological experiments. $K_v1.1$ and $K_v\beta1.1$ proteins were co-immunoprecipitated with either anti-$K_v1.1$ or anti-$K_v\beta1.1$ antibodies from oocytes metabolically labeled with [^{35}S]methionine/cysteine and were subjected to SDS–PAGE. The amount of $K_v\beta1.1$ that was co-precipitated with $K_v1.1$ by the $K_v1.1$ antibody was quantified and normalized to the amount of precipitated $K_v1.1$ protein. In three such experiments (see a representative experiment in Figure 4B, left panel), the normalized co-precipitated $K_v\beta1.1$ was 2.2 ± 0.09 ($p < 0.025$) fold larger in oocytes expressing Gβγ than in oocytes that did not express Gβγ. The co-immunoprecipitation with anti- $K_v\beta1.1$ antibody (Figure 4B, right panel) demonstrated that not all of $K_v\beta1.1$ was bound to $K_v1.1$, as more $K_v\beta1.1$ was precipitated with the $K_v\beta1.1$ antibody than co-precipitated with the $K_v1.1$ antibody. Taken together, the two biochemical types of analyses point to the probability that Gβγ expression increases the capacity of $K_v1.1$ to bind $K_v\beta1.1$. In calculating the amount of the $K_v\beta1.1$ protein, we referred only to the upper band of ~45 kDa, which corresponds to the predicted molecular weight; the nature of the ~35 kDa band is unknown to us (Levin *et al*., 1996). The bands corresponding to $K_v1.1$ protein (~57 kDa) in Figure 4B are not very prominent because the mRNA concentrations were minimal in order to be as close as possible to the physiological concentrations and at the same time to allow for reasonable resolution of the protein. Interestingly, when $K_v1.1$ and $K_v\beta1.1$ concentrations were increased up to 30-fold of those in an electrophysiological experiment, Gβγ expression had little or no effect on the $K_v\beta1.1$-binding capacity of $K_v1.1$, suggesting that in the presence of a large density of channel subunits, Gβγ loses its effect. The apparent Gβ co-precipitation with both antibodies (Figure 4B, lanes 3 and 6) is most probably an experimental artifact arising from the fact that this protein non-specifically binds to protein A–Sepharose (not shown).

The above biochemical analyses were done with whole oocyte homogenates and thus correspond mainly to channel proteins located in the cytoplasm and internal organelles, including the Golgi apparatus and endoplasmic reticulum (ER). The plasma membrane content of the channel proteins is <1% of total oocyte content (Levin *et al*., 1995). Therefore, it seems that Gβγ increased the $K_v\beta1.1$-binding capacity of $K_v1.1$ before reaching the plasma membrane. It has been shown in transfected mammalian cells that assembly of *Shaker* channel subunits takes place early in biosynthesis before reaching the plasma membrane (Shi *et al*., 1996; Nagaya and Papazian, 1997); this may be the case in oocytes. Consequently, one would predict that if there is a causative relationship between the effect of Gβγ on subunit assembly and its effect on inactivation of channels that are in the plasma membrane, the latter effect will be less prominent if Gβγ is not expressed together with the channel subunits, but later on, when much of the assembly of channels has already occurred. To address this prediction, we compared the effects of 2 days expression of Gβγ which was either co-injected with the channel subunits, as above, or injected 2 days following the injection of the channel subunits. Clearly, in two such experiments, the late expression of Gβγ did not result in increased inactivation; rather, the extent of inactivation decreased by a small but statistically significant extent (Figure 5A). At this stage, we do not understand the decrease in inactivation. To substantiate further the importance of the time at which the interaction between $G\beta_1\gamma_2$ and the channel occurs, we injected $G\beta_1\gamma_2$ 1 day prior to the injection of the channel subunits, in order to allow already synthesized $G\beta_1\gamma_2$ to be present from the very beginning of channel synthesis and assembly. As expected, in four experiments, the early expression of $G\beta_1\gamma_2$ had a larger effect on the extent of inactivation than that of co-expressed $G\beta_1\gamma_2$ (Figure 5B).

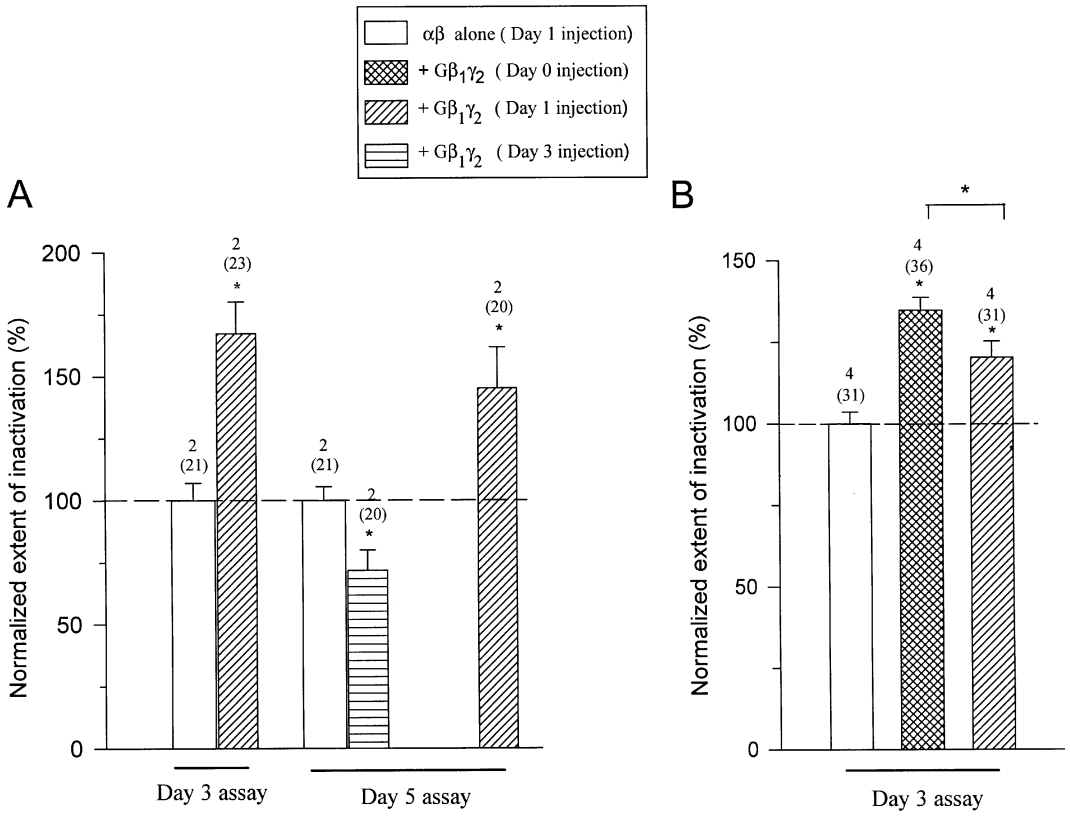


Fig. 5. The effects of late and early expression of Gβγ. (**A**) Comparison between the effects of late expression and co-expression of $G\beta_1\gamma_2$ on the extent of inactivation of the K^+ currents. Oocytes were injected on day 1 with either $K_v\beta1.1$ (β) and $K_v1.1$ (α) alone or co-injected with $G\beta_1\gamma_2$ and assayed electrophysiologically on day 3 and later on day 5; on both days of assay, the co-expressed $G\beta_1\gamma_2$ increased the extent of inactivation. On day 3, some of the oocytes that were injected on day 1 with $K_v\beta1.1$ and $K_v1.1$ alone were injected with $G\beta_1\gamma_2$ and assayed electrophysiologically on day 5; the late expression of $G\beta_1\gamma_2$ decreased the extent of inactivation (compared with oocytes injected on day 1 with $K_v\beta1.1$ and $K_v1.1$ alone). (**B**) Comparison between the effects of early expression and co-expression of $G\beta_1\gamma_2$. Oocytes that were either not injected or injected with $G\beta_1\gamma_2$ on day 0 were injected on day 1 with $K_v\beta1.1$ and $K_v1.1$ and assayed electrophysiologically on day 3. The early expression of $G\beta_1\gamma_2$ had a larger effect on the extent of inactivation. Numbers above the bars indicate number the of batches of oocytes; numbers in parentheses indicate the number of oocytes. $*p < 0.02$.

Discussion

Gβγ levels regulate the extent of inactivation of the $K_v1.1$–$K_v\beta1.1$ current

Our results show that co-expression of $G\beta_1\gamma_2$ subunits with a rat brain voltage-gated K^+ channel composed of $K_v1.1$ (α) and $K_v\beta1.1$ (β) subunits results in an increased fraction of the inactivating current component. Conversely, scavenging Gβγ by co-expression of C-βARK decreases the inactivating fraction. This fragment that binds Gβγ was demonstrated to attenuate the activation of Gβγ effectors and did not block Gα-mediated effector regulation (Inglese *et al*., 1994; Koch *et al*., 1994), suggesting that it could be a useful tool to distinguish Gα- from Gβγ-mediated events (Clapham and Neer, 1997). Also, we showed that Gβ can physically interact with both an intracellular domain of the $K_v1.1$ subunit, which is an integral membrane protein, and a domain of the $K_v\beta1.1$ subunit, which is a cytosolic protein. Taken together, these results strongly suggest that Gβγ is a primary mediator of the regulation of the $K_v1.1$–$K_v\beta1.1$ channel inactivation. This report is the first demonstration of a Gβγ-mediated regulation of a voltage-gated K^+ channel. Furthermore, the mechanism of Gβγ action is novel, as discussed below.

Gβγ as the physiological factor leading to disruption of all points of interaction between the channel and microfilaments relevant for inactivation

Previously, we showed that the $K_v1.1$–$K_v\beta1.1$ channel inactivates rapidly, but only partially, having a substantial non-inactivating current component that behaves identically to current through a channel composed of only $K_v1.1$ subunits (Levin *et al*., 1996; Jing *et al*., 1997). The extent of inactivation of the $K_v1.1$–$K_v\beta1.1$ current, but not its rate of inactivation, is dependent on interaction of the channel with microfilaments, and can be increased by depolymerization of microfilaments with DHCB. Part of the interaction with microfilaments is mediated via a PSD-95-like protein endogenous to the oocyte that interacts with the C-terminal end of $K_v1.1$; truncation of the C-terminal end results in increased inactivation. In this study, we showed that Gβγ occluded the effect brought about by disruption of the C-terminal interaction and could increase the extent of inactivation further, occasionally up to the level reached by the DHCB treatment. Hence, we have identified Gβγ as the physiological agent that is functionally identical to the disruption of all

the points of interaction between the channel and the microfilaments that bear upon inactivation. These effects on the macroscopic inactivation are consistent with two previously predicted (Levin *et al*., 1996), and now shown by us at the single channel level (Singer-Lahat *et al*., 1998), distinct modes of behavior of the $K_v1.1$–$K_v\beta1.1$ channel that underlie the whole-cell current. One mode is non-inactivating, identical to the mode of behavior of the $K_v1.1$ channel, giving rise to the macroscopic non-inactivating current component; the other mode is inactivating, giving rise to the inactivating current component. Interaction between the channel and microfilaments controls the equilibrium between the modes and shifts it towards the non-inactivating mode. In this context, we suggest that high Gβγ levels, leading to the disruption of this interaction, increase the number of channels in the inactivating mode.

Mechanism of Gβγ modulation

Part of the heterogeneity of voltage-gated K^+ channels arises from heteromultimerization of distinct pore-forming $K_v\alpha$ subunits with hydrophilic cytoplasmic $K_v\beta$ subunits. The cytoplasmic N-terminus of $K_v\alpha1$ (*Shaker*-related) contains a T1 domain which is composed of 'A' and 'B' regions (amino acids 38–71 and 72–143, respectively, in $K_v1.1$). The T1 domain specifies subfamily-specific assembly of the $K_v\alpha1$ subunits (Shen and Pfaffinger, 1996) and also delineates a region that is sufficient for interaction of $K_v\alpha1$ with $K_v\beta1$ subunits (Sewing *et al*., 1996; Yu *et al*., 1996; for a review see Scannevin and Trimmer, 1996). Within the 'B' domain, a sequence conserved among the $K_v\alpha1$ subunits (amino acids 119–127 in $K_v1.1$) has been mapped that is necessary for interaction with $K_v\beta1$ (Sewing *et al*., 1996). The $K_v\beta1$ subunit can be divided into two parts, a conserved core region and a variable N-terminal region. The latter region in $K_v\beta1.1$ (amino acids 1–72) contains an inactivating particle (Heinemann *et al*., 1994; Rettig *et al*., 1994) that can act in a ball-and-chain type of mechanism (Hoshi *et al*., 1990; Zagotta *et al*., 1990) to block the internal mouth of the channel rapidly upon depolarization and thereby to confer fast inactivation. The conserved core region is necessary and sufficient for interaction with $K_v\alpha1$ subunits (Xu and Li, 1997). In this study, by *in vitro* binding assays of GST fusion proteins to ^{35}S-labeled proteins, we showed that Gβγ directly interacts with the cytosolic N-terminal domain of $K_v1.1$, more specifically with the T1'B' domain itself, and with the variable N-terminal domain of $K_v\beta1.1$. As expected, $K_v\beta1.1$ protein also binds to the whole N-terminus of $K_v1.1$; however, it does not bind to the T1'B' domain itself, although this region contains the conserved sequence that has been shown to be necessary for $K_v\beta1$ interaction (Sewing *et al*., 1996). Apparently, the T1'B' domain is not sufficient for $K_v\beta1.1$ binding, as also suggested by functional assays that showed that T1'A' is also necessary (Sewing *et al*., 1996). Thus, it appears that the region of interaction of Gβγ with $K_v1.1$ overlaps part of the domain involved with $K_v\alpha1$–$K_v\alpha1$ and $K_v\alpha1$–$K_v\beta1$ assembly; however, it is different from the larger $K_v\beta1.1$ interaction domain of $K_v1.1$. The exact sequences in the T1'B' domain and the variable domain of $K_v\beta1.1$ that bind Gβγ remain to be mapped. Neither one contains a sequence motif QXXQR identified as a putative Gβγ-binding consensus sequence present in βARK and phospholipase Cβ which all interact directly with Gβγ (Koch *et al*., 1994; Chen *et al*., 1995). This motif is probably involved in modulation by Gβγ of Na^+ channel inactivation (Ma *et al*., 1997). However, the accumulating data from voltage-gated Ca^{2+} and GIRK channels indicate that this motif is not necessarily indicative of a functional Gβγ-binding site and that parts of protein that do not contain this motif can also bind Gβγ (for reviews see Dascal, 1997; Dolphin, 1998).

In co-immunoprecipitation experiments, we could not verify direct *in vivo* association of Gβγ with the $K_v1.1$–$K_v\beta1.1$ channel because Gβ bound non-specifically to protein A–Sepharose. However, the co-immunoprecipitation experiments with $K_v1.1$ antibody demonstrated that co-expression of Gβγ actually increases the $K_v\beta1.1$ binding capacity of $K_v1.1$, indicating that there is an equilibrium between free and $K_v1.1$-bound $K_v\beta1.1$ subunits and, in the presence of Gβγ, it is shifted towards the bound form. Indeed, concomitant co-immunoprecipitation experiments with $K_v\beta1.1$ antibody demonstrated that there are free $K_v\beta1.1$ subunits that are not bound to $K_v1.1$. A possible clue to the mechanism underlying this Gβγ effect is emerging from the *in vitro* studies showing that Gβγ interacts with both the $K_v\beta1.1$ and $K_v1.1$ subunits. The interaction of Gβγ with the part of the assembly domain on $K_v1.1$ does not seem to interfere with the interaction of $K_v\beta1.1$, as we could not detect any interference above a certain degree that appeared to arise solely from experimental artifacts. Indeed, the binding domains of Gβγ and $K_v\beta1.1$ do not match. Taken together, we visualize Gβγ existing in a complex with $K_v1.1$ and $K_v\beta1.1$. It is conceivable that Gβγ, through interaction with the T1'B' domain of $K_v1.1$ and the variable domain of $K_v\beta1.1$, serves as a sort of adhesive molecule that helps to attach $K_v\beta1.1$ to $K_v1.1$. However, one cannot exclude that the interaction of Gβγ is only with one component, i.e. either $K_v1.1$ or $K_v\beta1.1$. It is also conceivable that this effect takes place early in biosynthesis, most probably in the ER, where assembly of multimers of $K_v1.1$ and $K_v\beta1.1$ occurs (Scannevin and Trimmer, 1997). In support of this is the finding that injection of Gβγ mRNAs 2 days following the injection of the channel mRNAs did not increase the channel's inactivation; it is conceivable that during the first 2 days, most of the channel's biosynthesis and assembly occur. Correspondingly, injection of Gβγ mRNAs 1 day prior to the injection of the channel mRNAs, conceivably allowing the presence of already synthesized Gβγ proteins also at the onset of channel's assembly, increased the effect on inactivation.

The question arises as to the source of Gβγ available at the level of biosynthesis. Recent results suggest that Gβγ or Gβ can associate not only with plasma membranes but also with several subcellular compartments (Lin *et al*., 1992; Zwaal *et al*., 1996), including membranes of secretory granules (Muller *et al*., 1994) and the Triton X-100-insoluble pool enriched with actin (Carlson *et al*., 1986; for review see Neubig, 1994). In one case, free Gβγ subunits that trigger Golgi vasiculation were suggested (Jamora *et al*., 1997) to originate not from plasma membrane-bound heterotrimeric G proteins but rather from G proteins localized to the Golgi apparatus (e.g. $G\alpha_{i\text{-}3}$ that was shown to be localized on Golgi membranes; Stow

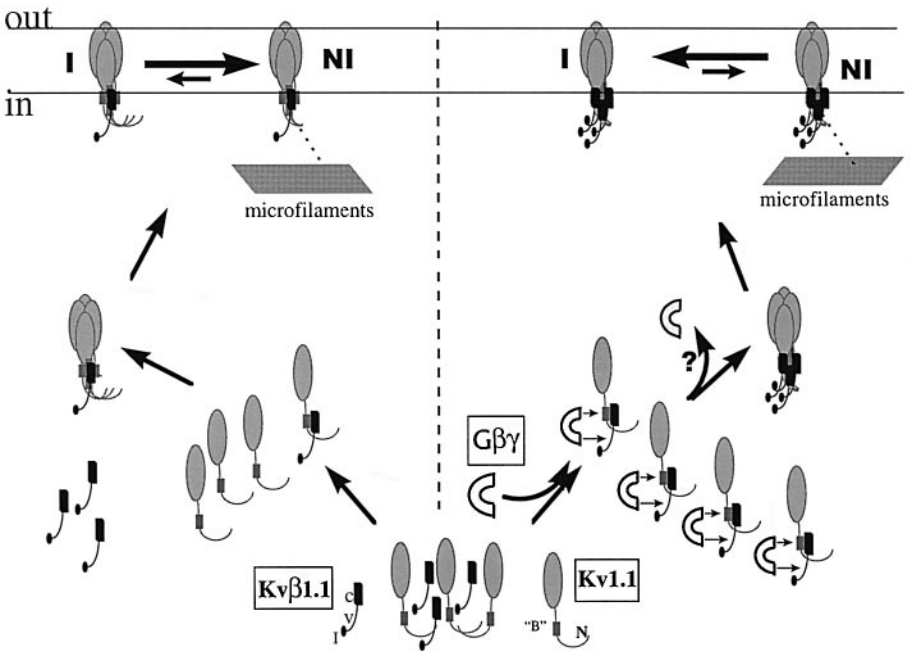


Fig. 6. Model for the Gβγ-mediated increase in extent of inactivation. A schematic diagram illustrating the postulated $K_v1.1$–$K_v\beta1.1$ channel assembly with (flow chart to the left) or without (flow chart to the right) co-expressed $G\beta_1\gamma_2$ and the resultant effects on channel interaction with microfilaments, leading to shifts in the equilibrium between two existing modes of behavior: fast inactivating (I) and non-inactivating (NI). Shown are the $K_v1.1$ subunits with their transmembrane core region and their N-terminus [having the T1'B' ('B') domain] and $K_v\beta1.1$ subunits with their core region (C) and variable (V) region that includes the inactivating ball domain (I). The model may not represent the actual stoichiometry of $K_v1.1$–$K_v1.1$ complexes; examples of extreme stoichiometries are shown.

et al., 1991) coupled to residents on Golgi membranes. In our case, since Gβγ probably binds directly to the channel subunits during the process of assembly, it is conceivable that Gβγ originates from trimeric G proteins localized on internal membranes. In such a case, a number of challenges are posed. Among these are the localization and identification of the internal membrane-bound trimeric G protein and possibly its upstream receptor. However, the possibility that Gβγ originates from plasma membrane-bound G proteins cannot be excluded, especially as it offers a mechanism for cross-talk between classical G protein signal transduction initiated at the cell surface and responding to extracellular signals, and the control of channel assembly that occurs in intracellular organelles and bears on channel function at the cell surface.

The next question that arises is how to interpret the correlation between the Gβγ-induced increase in the amount of $K_v\beta1.1$ bound to $K_v1.1$ subunits and the Gβγ-induced increase in the extent of inactivation of the channel. The determined stochiometry of $K_v\alpha1$ and $K_v\beta1$ is consistent with a $\alpha_4\beta_n$ model where n = 0–4, depending upon the relative concentration of $K_v1.1$ and $K_v\beta1.1$ (Xu *et al*., 1998). Also, one $K_v\beta1$ subunit that contains an inactivating particle, analogous to inactivating particles within $K_v\alpha1$ subunits (MacKinnon *et al*., 1993), is enough to confer inactivation (Xu *et al*., 1998). Taken together, a trivial rationalization of the Gβγ effect would be that the induced increase in the amount of $K_v\beta1.1$ bound to $K_v1.1$ results in more $K_v1.1$ tetramers that are assembled with at least one $K_v\beta1.1$, rendering more inactivating heteromultimers. However, this interpretation does not explain the observation that Gβγ co-expression and several hours of DHCB treatment are mutually occlusive with respect to their effect on inactivation. It is inconceivable that microfilament disruption during the few hours before the electrophysiological assay of functional plasma membrane channels can mimic the effect of Gβγ on channel assembly that probably takes place early in biosynthesis. Moreover, we previously showed that DHCB treatment does not affect the assembly of $K_v1.1$ and $K_v\beta1.1$ (Levin *et al*., 1996). Also, the possibility that DHCB treatment alters the population of channels inserted into the plasma membrane can be excluded since during the period of DHCB treatment, the current amplitudes did not change significantly (not shown); rather, it seems that DHCB affects channels that already reside in the plasma membrane. Thus, with this trivial interpretation being rejected, we looked for another scheme that will account for the Gβγ effect on inactivation.

In view of the results of this study, the above discussion and our previous knowledge (Levin *et al*., 1996; Jing *et al*., 1997), we propose the following scenario that describes the mechanism of inactivation of the $K_v1.1$–$K_v\beta1.1$ channel and also accounts for the effect of Gβγ (Figure 6). From a certain concentration ratio of $K_v\beta1.1$:$K_v1.1$, every tetramer of $K_v1.1$ is assembled with, at least, one $K_v\beta1.1$ subunit. Only one $K_v\beta1.1$ subunit per $K_v1.1$ tetramer is needed to render a potentially inactivating functional channel. However, whether this inactivation will be implemented is dependent on the interaction of the channel with the microfilaments: only detachment from microfilaments enables the inactivating particles on the $K_v\beta1.1$ subunit to confer inactivation. The more $K_v\beta1.1$ subunits per $K_v1.1$ tetramer, the more chances for the inactivation to be implemented, since the addition of $K_v\beta1.1$ subunits probably disrupts the interaction of the channel with microfilaments. The role of Gβγ is to enhance the binding of $K_v\beta1.1$ to $K_v1.1$, probably by the

generation of a ternary complex of Gβ–Gγ–K_v1.1–K_vβ1.1, and this probably takes place early in biosynthesis where K_v1.1–K_vβ1.1 complexes assemble (Nagaya and Papazian, 1997). It seems that the presence of Gβγ is not needed once the channel is in the plasma membrane, since channels formed from a saturating ratio of K_vβ1.1:K_v1.1 do not need expressed Gβγ in order to reach the maximal extent of inactivation (Figure 1C, left panel). In support of this notion, we could not observe any effects on K_v1.1–K_vβ1.1 channels of purified recombinant $G\beta_1\gamma_2$ proteins applied to the intracellular side of excised membrane patches (D.Singer-Lahat, I.Lotan and N.Dascal, unpublished results).

Since the effect of Gβγ occludes the effect arising from the disruption of the C-terminal interaction of K_v1.1 with microfilaments, one assumption would be that the interaction with microfilaments that is disrupted by Gβγ (conceivably involving either the N-terminus of K_v1.1 or K_vβ1.1, or both) is relevant for inactivation, and the effect of disrupting the C-terminal interaction arises merely from its impact on the N-terminal interaction. Once the N-terminal interaction is disrupted, the C-terminal interaction becomes irrelevant. Since Gβγ can bind to the T1'B' domain of K_v1.1 and to the variable N-terminal region of K_vβ1.1, the prediction is that the variable ability of different K_vα1–K_vβ1 channel complexes to inactivate can stem from variable sequences within these two regions.

Concluding remarks

This type of modulation by Gβγ of this voltage-gated K^+ channel is novel and differs from the well-documented Gβγ modulation of other ion channels such as the voltage-gated Ca^{2+} (for reviews see Wickman and Clapham, 1995; Clapham and Neer, 1997; Dolphin, 1998) and the inward rectifying K^+ GIRK channels (for review see Dascal, 1998). First, whereas the Gβγ modulation of K_v1.1–K_vβ1.1 affects the assembly of the channel complex that is probably taking place in early stages of biosynthesis and seems not to have a crucial role at the stage of functional channels in the plasma membrane, the Gβγ modulation of Ca^{2+} and GIRK channels occurs at the plasma membrane. Secondly, Gβγ does not seem to affect the activation of the K_v1.1–K_vβ1.1 channel, whereas the activation of Ca^{2+} and GIRK channels is affected by Gβγ. Such signal transduction by heterotrimeric G protein subunits affecting biosynthesis of an ion channel may underlie long-lasting changes in cell excitability. It is indeed different from the classical pathway occurring at the plasma membrane; however, it conforms with the established role of heterotrimeric G proteins in membrane traffic (e.g. see Bosmel and Mostov, 1992). Interestingly, the recently documented Gβγ modulation of brain Na^+ channels (Ma *et al*., 1997) appears synergistic to the above-described modulation of a brain K^+ channel; it does not affect activation of the channels, as well. Whereas Gβγ co-expression with Na^+ channels induces sustained currents and is consistent with a shift of channels from a rapidly inactivating to a slowly inactivating pool, Gβγ co-expression with the K^+ channels induces a decrease of the sustained current component and is consistent with a shift of channels from a slowly inactivating (C-type inactivation) to a fast-inactivating pool. Both of these changes (increased Na^+ influx and decreased K^+ efflux) should lead to an increase in excitability. Such a synergistic type of modulation may turn out to be a powerful means of regulation of neuronal excitability.

Materials and methods

Antisera, DNA constructs and mRNAs

K_v1.1 antiserum was generated against a 23 amino acid peptide that corresponds to the N-terminus of K_v1.1 (SGENADEASAAPGHPQDGSYPRQ), as described (Ivanina *et al*., 1994). $K_v\beta$ antiserum generated against GST fusion protein corresponding to the C-terminus of K_vβ1 was a generous gift from O.Pongs (Hamburg, Germany). K_v1.1, $K_v1.1_{S446A}$ and K_vβ1.1 cDNAs and their mRNA preparations were described previously (Levin *et al*., 1996). $K_v1.1_{S446A/K490s.c.}$ was prepared as described previously (Jing *et al*., 1997): Lys490 was substituted with a stop codon to delete the last six amino acids of the C-terminus of $K_v1.1_{S446A}$. K_vβ1.1 cDNA was a gift from O.Pongs. $G\beta_1$ and $G\gamma_2$ constructs were made by PCR to amplify the coding sequences of the corresponding DNAs (a gift from M.Simon), followed by subcloning into the *Eco*RI site of the pGEMHE vector. The C-βARK construct was made using a standard PCR technique to amplify the last 237 C-terminal amino acids of the rat βARK1 (a gift from R.J.Lefkowitz) in-frame with the first 15 N-terminal amino acids of src60. The C-βARK fragment was subcloned into pGEMHE vector. Constructs in pGEMHE were linearized with *Nhe*I and transcribed with T7 polymerase.

DNAs of K_v1.1 and K_vβ1.1 fragments to create GST fusion proteins were constructed by PCR amplification with Vent polymerase (New England Biolabs) using 100 ng of K_v1.1 or K_vβ1.1 plasmids as template for 25 cycles of 30 s at 95°C, 1 min at 55°C and 2 min at 72°C. For each fragment, unique forward and reverse primers were used, with the forward primer creating an *Eco*RI site followed by an initiation codon, and the reverse primer creating a *Not*I site followed by a stop codon. These fragments were cloned into *Eco*RI and *Not*I sites of pGEX-4T-1 (Amersham Pharmacia Biotech.). All PCR products were sequenced at Tel-Aviv University Sequencing Facility. Materials and enzymes for molecular biology were purchased from Boehringer Mannheim, Promega and MBI Fermentas.

Oocytes, drug treatments and electrophysiological recording

Frogs (*Xenopus laevis*) were maintained and dissected and their oocytes were prepared as described (Dascal and Lotan, 1992). Thus, for biochemical studies, oocytes were injected (50 nl/oocyte) with 100–200 ng/μl K_v1.1 and 1–6 μg/μl K_vβ1.1 mRNAs. For electrophysiological experiments, oocytes were injected (50 nl/oocyte) with 5–10 ng/μl K_v1.1 and 15–500 ng/μl (according to the β:α mRNA ratio stated in the text) K_vβ1.1 mRNAs. The concentrations of the injected mutant mRNAs deviated slightly from the above concentrations as they were adjusted to give current amplitudes similar to wild type. Aliquots of 100–200 ng/μl of Gβ, Gγ and C-βARK mRNAs were injected in both biochemical and electrophysiological experiments. Injected oocytes were incubated at 22°C for 1–4 days in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) supplemented with 1 mM $CaCl_2$, 2.5 mM sodium pyruvate and 50 μg/μl gentamycin, and then assayed either electrophysiologically or biochemically. DHCB treatment was done as described previously (Levin *et al*., 1996): oocytes were incubated in 40–60 μM of the drug for several hours prior to electrophysiological assay. A stock solution of DHCB was made in ethanol (kept at –20°C); control solutions always included 40–60 μM ethanol.

Two-electrode voltage clamp recordings were performed as described (Levin *et al*., 1995). To avoid possible errors introduced by series resistance, only current amplitudes up to 4 μA were recorded and, in a given experiment, the amplitudes of wild-type and mutant currents were similar. Currents were elicited by stepping the membrane potential from a holding potential of –80 to +50 mV for 200 ms. Current–voltage relationships were obtained by 200 ms depolarizing steps from –80 mV to the indicated voltages. Net current was obtained by subtraction of the scaled leak current elicited by a voltage step from –80 to –90 mV. Oocytes having a leak current of >3 nA/1 mV were discarded. Intervals of 20 s between each trace allowed for recovery from inactivation. The time course of the decay phase of the current was fitted with a sum of two exponential components. The slow component had a time constant of several tens of milliseconds, thus representing inactivation distinct from the fast inactivation of the fast component. The time constant of the fast component (τ_{inact}) is referred to in the text.

Metabolic labeling with [^{35}S]methionine/cysteine and immunoprecipitation

This was done essentially as described (Levin *et al*., 1995). Following injection of mRNA(s), 10–20 oocytes were incubated at 22°C for 2–4 days in NDE containing 0.2 mCi/ml [^{35}S]Met/Cys mix (Amersham). Homogenization was done in 150–300 µl of medium consisting of 20 mM Tris pH 7.4, 5 mM EDTA, 5 mM EGTA, 100 mM NaCl, 50 µg/ml phenylmethylsulfonyl fluoride (PMSF), 1 mM iodoacetamide, 1 µM pepstatin, 1 mM 1,10-phenanthroline (Sigma) supplemented with protein phosphatase inhibitors: 50 nM okadaic acid (Alomone Labs; Jerusalem), 0.5 mM vanadate (sodium orthovanadate; Almone Labs) and 50 mM KF (Sigma, St Louis, MO). Yolk was removed by centrifugation at 1000 *g* for 10 min at 4°C. After addition of Triton X-100 to a final concentration of 4%, followed by centrifugation at 8000 *g* for 15 min at 4°C, antiserum was added to the supernatant for 16 h. After 1 h incubation with protein A–Sepharose, immunoprecipitates were pelleted and washed four times with immunowash buffer (150 mM NaCl, 6 mM EDTA, 50 mM Tris pH 7.5, 0.1% Triton X-100); the final wash contained no Triton X-100. Samples were boiled in SDS–gel loading buffer and electrophoresed on an SDS–8% polyacrylamide gel together with standard molecular mass markers (29–205 kDa).

Western blotting

Twenty to thirty oocytes previously assayed electrophysiologically were homogenized and proteins processed until SDS–gel electrophoresis, as described above. In parallel, 20 oocytes of the same batch were homogenized and processed similarly except that instead of antibody, ethanol was added for 1 h at –20°C followed by 15 min centrifugation at 8000 *g* to pellet the proteins. After electrophoretic transfer to PVDF membranes, the resulting blots were blocked in 0.15 mM NaCl, 10 mM Tris–HCl, pH 8 (TBS) containing 3% low fat milk and 0.1% Tween-20 (blot solution) supplemented with 0.1% Na azide, incubated in K_v1.1 antibody diluted 1:500 in blot solution for 1 h, and washed five times in blot solution for 50 min total. Blots were then incubated in horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Bio-Rad; 1:5000 dilution in blot solution) for 30 min and then washed in blot solution five times for 50 min total; the last washing without milk. The blots were then incubated in substrate for enhanced chemiluminescence (ECL).

Binding of the GST fusion proteins to ^{35}S-labeled proteins

[^{35}S]Met/Cys-labeled Gβ, Gγ and K_vβ1.1 were translated on the template of *in vitro* synthesized RNAs using a rabbit reticulocyte translation kit (Promega) according to the manufacturer's instructions. The fusion proteins were synthesized and extracted from *Escherichia coli* according to the pGEX-4T-1 manufacturer's instructions (Amersham Pharmacia Biotech.). The protein concentration was estimated using the Bio-Rad protein assay kit (Munich, Germany). Purified GST fusion proteins (5–10 µg) or purified GST (~10 µg) were incubated with 5–15 µl of the lysate containing ^{35}S-labeled Gβ and Gγ or K_vβ1.1 in 500 µl of phosphate-buffered saline (PBS) with 0.05% Tween-20, for 2 h at room temperature, with gentle rocking. Then the GST fusion proteins were immobilized on gluthatione–Sepharose beads (Amersham Pharmacia Biotech, 30 µl of beads were added) for 30 min at 4°C and washed four times in 1 ml of PBS with 0.05% Tween-20. Following washing, the GST fusion proteins were eluted with 20 mM reduced gluthatione in 30 µl of elution buffer (120 mM NaCl, 100 mM Tris–HCl, pH 8) and analyzed by SDS–PAGE.

Quantification of labeling intensities and generation of digitized PhosphorImager scans

Gels were dried and placed in a PhosphorImager (Molecular Dynamics) cassette for ~1 day. Using the software ImageQuant, a digitized scan was derived, and relative intensities of protein bands were estimated quantitatively by the software ImageQuant as described (Levin *et al*., 1995).

Statistical analysis

Data are presented as means ± SEM; *N* denotes number of frogs assayed, *n* denotes the number of oocytes assayed. *t*-tests were used to calculate the statistical significance of differences between two populations.

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