Two classes of regulatory subunits coassemble in the same BK channel and independently regulate gating

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High resolution proteomics increasingly reveals that most native ion channels are assembled in macromolecular complexes. However, whether different partners have additive or cooperative functional effects, or whether some combinations of proteins may preclude assembly of others are largely unexplored topics. The large conductance Ca²⁺-and-voltage activated potassium channel (BK) is well-suited to discern nuanced differences in regulation arising from combinations of subunits. Here we examine whether assembly of two different classes of regulatory proteins, β and γ, in BK channels is exclusive or independent. Our results show that both γ₁ and up to four β₂-subunits can coexist in the same functional BK complex, with the gating shift caused by β₂-subunits largely additive with that produced by the γ₁-subunit(s). The multiplicity of β;γ combinations that can participate in a BK complex therefore allow a range of BK channels with distinct functional properties tuned by the specific stoichiometry of the contributing subunits.

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Proteomic\textsuperscript{1,2} and functional\textsuperscript{2-6} studies have revealed many partners that interact with BK channels, some of which are known to confer distinct tissue-specific functional properties on the BK pore-forming subunit. BK channels are dually activated by membrane depolarization and increases in intracellular [Ca\textsuperscript{2+}] (refs 7-9). The minimal functional unit of a BK channel is a homotetramer of pore-forming \(\alpha\)-subunits (Fig. 1a), each containing intrinsic voltage-sensing and Ca\textsuperscript{2+} -sensing domains. However, BK channels may also contain any of two different families of regulatory proteins, \(\beta\) and \(\gamma\), which help define tissue-specific functional properties of a BK complex. The four members of the \(\beta\) family (\(\beta_1-\beta_4\)) and the four members of the recently discovered \(\gamma\) family (\(\gamma_1-\gamma_4\)) differentially regulate BK function, influencing Ca\textsuperscript{2+} -dependence of activation\textsuperscript{2,5,6,10,11}, current inactivation\textsuperscript{1,5,12,13} and even pharmacology\textsuperscript{5,14}.

\(\beta\) - and \(\gamma\) -subunits are unrelated proteins with very different predicted structural topology. \(\beta\)-subunits contain two transmembrane (TM) segments linked by an extracellular loop bridged by multiple disulfide linkages and intracellular C and N termini\textsuperscript{15}; \(\gamma\)-subunits contain a single TM segment, a cytosolic C terminus, and an extracellular N terminus with a large leucine-rich repeat-containing motif\textsuperscript{6,16} (Fig. 1b). Specific structural information for \(\beta\) - and \(\gamma\) -subunits is not available. For \(\beta\)-subunits, potential positions of TM segments in relation to \(\alpha\)-subunit TM segments have been proposed\textsuperscript{17-19} (Fig. 1c). Individual BK channels can contain 0–4 \(\beta\)-subunits, with each subunit contributing in an

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energetically independent fashion to shift BK gating\(^\text{20}\). For \(\gamma\)-subunits, both the position in the channel complex and the \(\alpha:\gamma\) stoichiometry remain unknown. In heterologously expressed \(\alpha + \gamma 1\) channels, the \(\gamma 1\)-induced gating shift occurs in an all-or-none manner, consistent with an elementary functional unit of \(\gamma 1\) (for example, monomer, dimer and tetramer) being sufficient to produce the full effect\(^\text{18}\). Here to determine whether different types of regulatory subunits coassemble in the same BK channels, we take advantage of the distinctive functional properties conferred on BK channels by \(\beta 2\) and \(\gamma 1\)-subunits. Using ensemble and single molecule approaches we report that \(\gamma 1\) and 1–4 \(\beta 2\)-subunits can be simultaneously present in the same BK channel and independently contribute to modulation of BK function.

**Results**

**\(\beta 2\) and \(\gamma 1\)-subunits coassemble in the same BK channel.** The \(\gamma 1\)-subunit produces a remarkable negative shift of 120–140 mV in the voltage-range of BK channel activation either in the presence or absence of \(\text{Ca}^{2+}\)\(^\text{2}\) (Fig. 1e,h,i). While BK channels composed of \(\alpha\) alone require at least 10 \(\mu\text{M}\) \(\text{Ca}^{2+}\) to show appreciable open probability over physiologically relevant voltages, (Fig. 1h,i), \(\alpha + \gamma 1\) channels show a similar probability in the total absence of intracellular \(\text{Ca}^{2+}\) (Fig. 1e,h). The ability of \(\gamma 1\) to shift BK gating in 0 \(\text{Ca}^{2+}\) contrasts with the absence of a gating shift produced by \(\beta 2\) (Fig. 1h) under the same conditions\(^\text{22}\).

The most readily identifiable effect of the \(\beta 2\)-subunit is essentially complete inactivation that occurs following channel activation (Fig. 1f). Inactivation arises from the cytosolic N terminus of the \(\beta 2\)-subunit\(^\text{4,5,23}\). Since BK channels can contain 1–4 \(\beta 2\)-subunits, each acting independently, single \(\alpha + \beta 2\) channels exhibit one of four distinct inactivation rates\(^\text{20}\). The inactivation time constant \((\tau\text{inact})\) therefore provides a measure of \(\beta 2\)-subunit stoichiometry within either a channel population or individual channels\(^\text{20}\). Although \(\beta 2\) produces little gating shift at 0 \(\text{Ca}^{2+}\)\(^\text{23}\) (Fig. 1h), gating of \(\alpha + \beta 2\) channels at 10 \(\mu\text{M}\) \(\text{Ca}^{2+}\) is shifted about 40 mV leftward compared with \(\alpha\) channels\(^\text{5,20}\) which is more clearly observed using a non-inactivating \(\beta 2\) variant (\(\beta 2\Delta\text{NT}\)) (Fig. 1i). Thus, for \(\alpha + \beta 2\) channels, voltage steps up to +180 mV only weakly activate BK currents at 0 \(\text{Ca}^{2+}\) (Fig. 1f, left), while 10 \(\mu\text{M}\) \(\text{Ca}^{2+}\) produces robust activation of an inactivating current (Fig. 1f, right), whose \(\tau\text{inact}\) approaches a limiting value of \(\approx 20\) ms (Fig. 1i), indicating an average of 4 \(\beta 2\)-subunits/channel in the population\(^\text{20}\). Another physiologically relevant property of inactivating channels, the voltage dependence of steady-state inactivation (SS-inactivation), is also an indicator of the presence of \(\beta 2\)-subunits. During exposure of \(\alpha + \beta 2\) channels to 10 \(\mu\text{M}\) \(\text{Ca}^{2+}\), the availability of non-inactivated channels to open is very low at voltages below –50 mV (Fig. 2a,c) with half-channel availability \((V_\text{50})\) at about –110 mV. Together, the distinctive effects of \(\beta 2\) and \(\gamma 1\)-subunits provide useful signatures to verify the presence of \(\beta 2\) and \(\gamma 1\) in BK channels resulting from the coexpression of \(\alpha + \beta 2 + \gamma 1\).

We therefore coexpressed \(\gamma 1 + \beta 2\) subunits with \(\alpha\) at relative mole fractions of message that would produce full effects of either \(\gamma 1\) or \(\beta 2\) alone\(^\text{20,21}\). The coexpression of \(\alpha + \beta 2 + \gamma 1\) subunits results in a prominent inactivating outward current even with 0 \(\text{Ca}^{2+}\) (Fig. 1g, left), similar to currents resulting from the expression of \(\alpha + \beta 2\) subunits when activated by 10 \(\mu\text{M}\) \(\text{Ca}^{2+}\) (Fig. 1i). The complete inactivation of currents obtained after coexpression of \(\alpha + \beta 2 + \gamma 1\) subunits indicates that essentially all channels contain \(\beta 2\)-subunits. Furthermore, that in such patches the voltage dependence of activation at 0 \(\text{Ca}^{2+}\) is shifted more than –120 mV (Fig. 1h) is diagnostic for the presence of \(\gamma 1\). When the same patch is activated with 10 \(\mu\text{M}\) \(\text{Ca}^{2+}\), essentially no current is observed (Fig. 1g, right) which suggests that \(\alpha + \beta 2 + \gamma 1\) channels are constitutively inactivated with 10 \(\mu\text{M}\) \(\text{Ca}^{2+}\) at –160 mV. Indeed, the fractional availability of \(\alpha + \beta 2 + \gamma 1\) currents at 0 \(\text{Ca}^{2+}\) (Fig. 2b) exhibits a voltage dependence very similar to that observed with 10 \(\mu\text{M}\) \(\text{Ca}^{2+}\) for \(\alpha + \beta 2\) currents (Fig. 2c–d). The markedly leftward-shifted steady-state inactivation curve of \(\alpha + \beta 2 + \gamma 1\) channels also confirms that both \(\beta 2\) and \(\gamma 1\)-subunits can coassemble in the same BK channels.

**\(\beta 2\) and \(\gamma 1\) occupy distinct positions in the BK channel complex.** We next wondered whether channels containing both \(\beta 2 + \gamma 1\) subunits can contain a full set of four \(\beta 2\)-subunits. We imagined two kinds of assembly scenarios: (1) an independent model (Fig. 3a), where the presence of \(\gamma 1\)-subunits does not hinder the ability of four \(\beta 2\)-subunits to fully populate a BK channel (\(\beta 2\) and \(\gamma 1\) occupy different positions), or (2) an occlusive model (Fig. 3b), where the presence of \(\gamma 1\) excludes the assembly of \(\beta 2\)-subunits (\(\beta 2\) and \(\gamma 1\) occupy overlapping positions). These two models can be tested by examination of the \(\tau\text{inact}\) arising from a set of single \(\alpha + \beta 2 + \gamma 1\) channels obtained under conditions in which relative \(\beta 2\) subunit expression varies\(^\text{20}\). If the independent model is valid, \(\gamma 1\)-containing inactivating single channels should exhibit four distinct \(\tau\text{inact}\) (ref. 20), while the finding of less than four
- Consecutive traces of current recorded at 0 Ca\textsuperscript{2+} injection ratios (given above each set of traces). For each example, five representing two distinctive assembly models: where s\textsuperscript{1}–s\textsuperscript{n}.

- Mean values (\textit{t}inact) of each Gaussian component are defined by the slowest component, the \textit{t}inact distribution is better fit by a four-component Gaussian distribution (Fig. 3g,h), Supplementary Fig. 2), indicating that channels containing the \textit{γ}1-induced effect can also contain one, two, three or four \textit{β}2-subunits. In the case of the three-component Gaussian, if the constraint on \textit{s}d is relaxed, the best fit is reached with the fastest component having the largest s.d. of any component, suggestive that this fast component in fact arises from two populations. The superior fit of the four-component Gaussian supports the existence of individual channels containing 4 \textit{β}2 and at least one \textit{γ}1-subunit indicating that both types of auxiliary subunits occupy independent positions in the channel complex (Fig. 3a).

- \textit{β}2 and \textit{γ}1 independently contribute to BK gating shifts. Both \textit{β}2 and \textit{γ}1 produce leftward shifts in BK gating, but their effects are likely mediated by different mechanisms. Whereas the \textit{γ}1-effect can be explained by stronger coupling between the voltage-sensor movement and channel activation\textsuperscript{4}, the effects of \textit{β}2 appear more complex\textsuperscript{22–26}. We asked whether \textit{γ}1 and \textit{β}2 effects might be additive or occlusive. For better elucidation of the \textit{β}2 effects, we compared the gating shift resulting from the coexpression of \textit{γ}1 + \textit{β}2\textit{ΔNt} versus that produced by each construct separately when coexpressed with BK-\textit{α}-subunits (Fig. 4). The \textit{V}0\textsubscript{s} arising from the simultaneous presence of \textit{γ}1 and \textit{β}2\textit{ΔNt} approximately reflects the sum of the independent effects of \textit{γ}1 and \textit{β}2\textit{ΔNt} alone. These results indicate that, whatever the underlying molecular mechanism of the \textit{V}0\textsubscript{s} shift produced by the \textit{γ}1-subunit, it is predominantly energetically independent of that produced by the \textit{β}2-subunit. Furthermore, there is no inhibitory allosteric coupling between the auxiliary subunits themselves.

- Other \textit{β}-subunits also coassemble with \textit{γ}1 in BK channels. Can other \textit{β}-subunits also coassemble with \textit{γ}1 in BK channels?
An earlier report suggested that the presence of β1-subunits may occlude the ability of γ1 to produce its gating shift\(^2\). Since the overexpression of one subunit might influence the successful expression of another, we used proportions of RNA for each subunit similar to those used in testing γ1 + β2 coassemblies with α-subunits. Using a β1 construct in which its N terminus was replaced by the β2-N terminus (β1/β2N) so that inactivation reports the presence of β1, we found that all BK channels resulted from coexpression of α + β1/β2N + γ1 simultaneously contain both types of regulatory subunits (Supplementary Fig. 3).

**Discussion**

The present work unambiguously shows that two different types of non-pore-forming regulatory subunits, β2 and γ1, can coassemble in the same functional BK channel and independently regulate channel function. The α + β2 + γ1 combination generates a BK channel with novel functional properties, which subtly change depending on the stoichiometry of β2 in the multimeric complex. In a normal cellular environment, the simultaneous presence of β2 + γ1 in BK channels might effectively remove them from availability for activation especially when intracellular calcium is increased. However, at 0 Ca\(^{2+}\), such channels would not be fully inactivated (Fig. 2d): the ratio of coassembly would also likely apply to other members of the BK multimeric complex. In a normal cellular environment, the α + β2 + γ1 subunits are simultaneously present in all single-cell type, reported message levels of both β2 and γ1-subunits in some tissues\(^11,27\) support the possibility that β- and γ-subunits will copartner in at least some cells. For any cells which may express both a β2- and a γ1-subunit, our results establish that these two distinct regulatory partners of BK channels can simultaneously and independently contribute to modulation of BK function and do not appear to hinder the assembly of each other into a channel. The possibility of coassembly would also likely apply to other members of the β and γ families, as supported by our results with β1 + γ1 subunits.

Our findings highlight not only the critical importance of defining the identity of protein partners in native multimeric complexes within a cell or a specific cell location, but also the importance of understanding how individual contributions of distinct regulatory components and their stoichiometry can define fundamental properties of the complex.

**Methods**

**Constructs.** Primary constructs were mouse α (SLO1) (ref. 9), human LRRC26 (γ1) (ref. 11) and human β2 (ref. 23). In some experiments β2AN (the first 33 amino acids were removed from the β2-N-terminal) or β1/β2AN (first 43 amino acids from the β2-N terminus replaced the first 11 amino acids of the β1-N terminus) were used.

**Oocyte expression.** Stage IV Xenopus laevis oocytes were used for channel expression. The complementary RNAs (cRNAs) of all constructs were prepared at approximately 1 μg/μL. For macroscopic recordings, cRNA mixes containing (molar ratio): mSLO1 alone, mSLO1 + hβ2 (1:6.5), mSLO1 + hLRRC26 (1:4) or mSLO1 + hβ2 + hLRRC26 (1:6.5:4), were diluted 1:5 before injection. For single channel recordings, mSLO1-hβ2LRRC26 (1:0.65:4) and (1:0.16:4) were diluted 1:20–1:100 before injection. β2AN or β1/β2AN cRNAs were used at the same molar ratio as β2 in some experiments. Oocytes were used 2–5 days after injection, except for single channel recordings in which they were used 1–2 days after injection. Maintenance of frogs and isolation of oocytes following procedures contained both types of regulatory subunits (Supplementary Fig. 3).

**Electrophysiology.** Borosilicate glass capillaries (1B150F-4, World Precision Instruments) were pulled to diameters resulting in resistances of 1–2 or 5–6 MΩ for macroscopic and single channel recordings, respectively. Pipettes were coated with Sylgard 184 (Dow Chemical Corp.) and fire-polished. Currents were recorded in the inside-out patch configuration using an Axopatch 200B amplifier (Molecular Devices) and the Clampex program from the pClamp software package (Molecular Devices). Gigaohm seals were formed in frog Ringer (in mM, 115 NaCl, 2.5 KCl, 1.8 CaCl\(_2\), 10 HEPES, pH 7.4) and, after patch excision, moved into flowing test solutions. The pipette/extracellular solution was in (mM): 140 K-methanesulfonate, 20 KOH, 10 HEPES, 2 MgCl\(_2\), pH 7.0. Test solutions of different Ca\(^{2+}\) were prepared by 140 mM methanesulfonate, 20 mM KOH, 10 mM HEPES with pH adjusted to 7.0 (ref. 28). N-(2-hydroxyethyl)tris(2-aminoethyl) triacetate acid (HEDTA) was replaced for 10 μM Ca\(^{2+}\) and 5 mM ethylene glycol-bis(2-aminoethyl)ether-N,N,N’N’- tetraacetate (EGTA) for 0 μM Ca\(^{2+}\) solutions. The 10 μM Ca\(^{2+}\) solution was titrated to appropriate pCa with Ca-MES and calibrated against solutions of defined Ca\(^{2+}\) concentrations (World Precision Instruments) using a Ca-sensitive electrode. Test solutions were applied directly to patches via a large bore micropipet containing multiple independent solution lines. All experiments were at room temperature (~22–25°C).

**Data analysis.** Analysis were made using Clampfit (Molecular Devices). For non-inactivating currents, conductance values (G) were obtained from the tail currents, while for inactivating currents the peak current was used. G-V data sets were fitted with a single Boltzmann function: \(G(V) = \frac{G_{\text{max}}}{1 + e^{\frac{V - V_{\text{h}}}{C_0}}}\), where \(G_{\text{max}}\) represents the amplitude, mean-\(t_{\text{inact}}\) and s.d. of each component, respectively, with \(n = 1\) for the parameters of the slowest inactivating component, \(n = 2\) for the second slower and so on. Since each measured \(t_{\text{inact}}\) represents the mean of an exponentially distributed population, the s.d. for the average of those grouped in the \(t_{\text{inact}}\)-distribution should be larger than for faster components, such that \(s_{\text{1}} < s_{\text{2}} < s_{\text{3}}\). Fitting to the sum of three or four Gaussian functions was first made allowing free variation of all fitting parameters. However, in both cases the rank of s.d. do not follow the expected criteria mentioned before: the four-component fit yields a very narrow third-component (very small \(s_{\text{3}}\)) while the three-component fit results with the fastest component having the largest s.d. \(s_{\text{3}}\) among all components (Supplementary Fig. 2a–b). Two factors may contribute to such deviations: first, that the numbers of entries in the histogram are insufficient to accurately define all aspects of each component and, second, that some components may actually arise from multiple components. We then constrained the s.d. in both cases to fulfil the expected criteria \((s_{\text{1}} < s_{\text{2}} < s_{\text{3}})\) (see results in Fig. 3g–h). Furthermore, we took advantage of the idea that inactivation results from the independent movement of each inactivation domain to the central cavity of the pore\(^21\). Based on this latter consideration, in some cases we also constrained all mean values of each component to be dependent on the slowest component (Supplementary Fig. 2c–d). All cases yield better fits using four than three-component Gaussian distributions.

**Chemicals.** Salts and buffers were obtained from Sigma.

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### Author contributions
V.G.-P. and C.J.L designed research, analysed data and prepared the manuscript. V.G.-P collected the data. X.-M.X. contributed new reagents/analytical tools.

### Additional information
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