

Determination of the Stoichiometry between α - and γ 1 Subunits of the BK Channel Using LRET

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ABSTRACT Two families of accessory proteins, β and γ , modulate BK channel gating and pharmacology. Notably, in the absence of internal Ca^{2+} , the γ 1 subunit promotes a large shift of the BK conductance-voltage curve to more negative potentials. However, very little is known about how α - and γ 1 subunits interact. In particular, the association stoichiometry between both subunits is unknown. Here, we propose a method to answer this question using lanthanide resonance energy transfer. The method assumes that the kinetics of lanthanide resonance energy transfer-sensitized emission of the donor double-labeled α/γ 1 complex is the linear combination of the kinetics of the sensitized emission in single-labeled complexes. We used a lanthanide binding tag engineered either into the α - or the γ 1 subunits to bind Tb^{3+} as the donor. The acceptor (BODIPY) was attached to the BK pore-blocker iberiotoxin. We determined that γ 1 associates with the α -subunit with a maximal 1:1 stoichiometry. This method could be applied to determine the stoichiometry of association between proteins within heteromultimeric complexes.

BK channels are homotetramers of the pore-forming α -subunit, which is broadly expressed in mammal tissues and its distinctive physiological function is to dampen the effects of the cytosolic increase of Ca^{2+} concentration due to the opening of voltage-dependent Ca^{2+} channels (1–4). Although BK channels are coded by a single gene (*KCNMA1*), they display a remarkable functional diversity, largely due to their interaction with accessory subunits. Two families of BK channel accessory subunits, the β -family (β 1– β 4) (5–8) and the γ -family (γ 1– γ 4) (9,10), confer new and physiological relevant phenotypes to the BK channel. Within the γ -family, the most remarkable effects are produced by the γ 1 subunit (9). This accessory subunit greatly increases the allosteric coupling between voltage sensors and pore, shifting the conductance-voltage (G-V) curve >150 mV to the left along the voltage axis (9,10).

Although the structure of the *Aplysia* BK channel was determined by the cryo-electron microscopy technique (11,12), little is known about the detailed structure of the accessory subunits beyond their secondary structure and putative membrane topology (10,13). Regarding the stoichiometry between the α -subunit and its accessory subunits, it is known that the tetramer formed by α can hold from one to

four β 1 or β 2 subunits (14). However, the stoichiometry between α - and any of the γ -subunits is unknown at present. Interestingly, the γ 1 subunit displays an all-or-none effect on the BK channel. However, there is not a definite test of how many γ 1 subunits are necessary to cause this effect (15).

Lanthanide resonance energy transfer (LRET) technique, together with symmetric nanopositioning system (SNPS) analysis (16), have been previously used to study conformational changes in ion channels in response to a stimulus like, for example, a change in membrane voltage (16,17). LRET/SNPS calculates the positions of terbium ions chelated by lanthanide binding tag (LBT) motifs (18), which are strategically inserted in different positions of the protein of interest. In the case of β 1, LRET/SNPS was used to determine the structural rearrangements due to the interaction between the α - with β 1 subunits (19). Given the tetrameric structure of BK, the LBT-labeled channel contains four LBT- Tb^{3+} donors interacting with a single toxin-BODIPY acceptor. Two alternative strategies were used to study the ($\alpha + \beta$ 1)BK channel complex (19). In the first approach, the α -subunits were LBT-labeled to explore the Tb^{3+} positions in the absence and the presence of β 1 subunit. In the second strategy, β 1 was LBT-labeled to determine its position relative to the α -subunit. In both cases, because the acceptor is located outside the center of symmetry of the channel, there are four different donor-acceptor distances. Therefore, one way to analyze the LRET/SNPS results is

Submitted February 12, 2018, and accepted for publication April 5, 2018.

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Editor: William Kobertz.

<https://doi.org/10.1016/j.bpj.2018.04.008>

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to describe the decay of the luminescence of the donors in the presence of the acceptor, the sensitized emission (SE), as a set of four different time constants instead of determining Tb^{3+} positions.

Unlike Förster resonance electron transfer, in LRET the acceptor lifetime is several orders-of-magnitude shorter than that of the donor (20), implying that during the time course of donor decay the acceptor can emit and be reexcited many times. This enables the total SE of the acceptor to be the linear combination of the SEs for each donor-acceptor pair. In fact, the SNPS method (16) is based on this idea; the SE of the acceptor is the linear combination of the time constants of the four donors because stoichiometrically each one of the donors is equally represented. Here we extended this idea to a heteromultimeric protein complex by assuming that the kinetics of SE of the double-labeled $\alpha/\gamma 1$ complex is the linear combination of the kinetics of the SE of the single-labeled complexes.

We used two LBT insertion sites in the extracellular face of the α -subunit (Fig. 1 A). One is in the BK N-terminus (between the D15 and S16 residues) and the second is in the C-terminal of the S1 transmembrane segment (between the S134 and the S135). Both constructs were expressed in *Xenopus laevis* oocytes and tested using the patch-clamp technique to verify their degree of expression and functionality. Both α -LBT constructs displayed robust ionic currents and their activation by voltage was similar to that of the wild-type (wt) α , as previously reported (Fig. S1, A–C, top) (19). In addition, the $\gamma 1$ subunit was coexpressed with the α (wt) and each α -LBT construct to confirm that $\gamma 1$ was shifting the G–V curve of the α -LBT channels to the left, as the wt subunit (Fig. S1, A–C, middle). The results showed a large leftward shift of the G–V curve for the α (wt) and the α -LBT NT but not for α -LBT S1 construct (Figs. S1, D–F, and 1 B). Below, we discuss this anomalous behavior of $\gamma 1$ when coexpressed with the α -LBT S1 construct.

In $\gamma 1$, LBT was inserted in the N-terminal of the leucine-rich repeats domain, between the D42 and S43 residues (Fig. 1 A). Robust currents were recorded when this construct was coexpressed with α (wt) and both α -LBT constructs (Fig. S1, A–C, bottom). $\gamma 1$ -LBT produces similar effects than those produced by $\gamma 1$ (wt) (Figs. S1, D–F, and 1 B), suggesting that the insertion of the LBT did not change the structure and function of the $\gamma 1$.

All LRET experiments were performed at a holding membrane potential of -80 mV using the two-electrode voltage-clamp technique. The intracellular Ca^{2+} concentration of *Xenopus* oocytes is submicromolar (20), such as at -80 mV all (α)BK channels are in the closed configuration. Indirect evidence indicates that $\gamma 1$ does not change the voltage-sensor workings (10), whereby we assumed that, like (α)BK channels, voltage-sensor domains of ($\alpha + \gamma 1$) BK channels are also in the resting state at -80 mV.

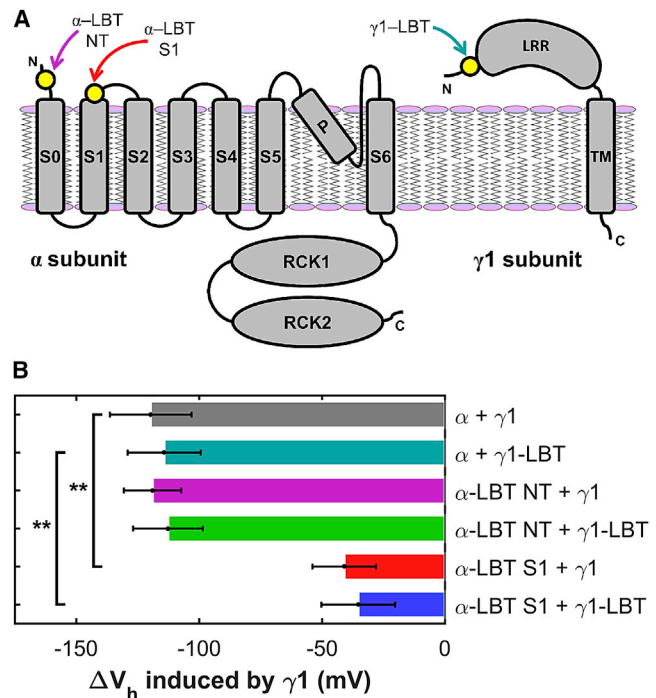


FIGURE 1 BK half-activation voltage shifts induced by $\gamma 1$ (wt) and $\gamma 1$ -LBT construct. (A) Cartoon is given depicting LBT insertion sites in α - and $\gamma 1$ subunits. (B) Changes in (G/G_{max}) –V half-activation voltages (ΔV_h) is induced by $\gamma 1$ wt or $\gamma 1$ -LBT on BK α (wt) and α -LBT NT and S1 constructs. Bar and error bars lengths represent the mean and the mean \pm SE ($N = 6$ –13; see Fig. S1), respectively; $**p < 0.01$ (two-tailed Student's *t*-test). To see this figure in color, go online.

Donor-only emission (DOE) signals from LBT-labeled constructs were obtained as described in the Supporting Materials and Methods. Luminescence decays recorded after a brief light flash were obtained in all cases (Fig. S2, A–C, top, colored traces), whose slowest component had a time constant ~ 2.4 ms, which is absent in oocytes expressing nonlabeled α - and γ -subunits (Fig. S2, A–C, top, gray traces). This slowest component has the expected time constant of the decay of excited Tb^{3+} bound to LBT in the absence of the acceptor (τ_{DOE}) (16,18,19). All LBTs constructs tested showed $\tau_{DOE} > 2.25$ ms, indicating that the LBTs are properly folded after insertion into either protein (Fig. 2 A; Table S1).

In the presence of 500 nM iberiotoxin-BODIPY, the time-course of the SE of the BODIPY was recorded as described in Supporting Materials and Methods. The acceptor position, out of the symmetry axis of the channel, determines that there are four different donor-acceptor distances and therefore the SE decay may contain up to four exponential decays (16,19,21,22). (α -LBT + $\gamma 1$)BK channels SE decays, like those in Fig. S2, A–B (bottom), were SNPS-fitted by the equation (19,23):

$$I(t) = W_{\alpha} \sum_{i=1}^4 k_{\alpha,i} e^{-\frac{t}{\tau_{\alpha,i}}} + U(t) + c, \quad (1)$$

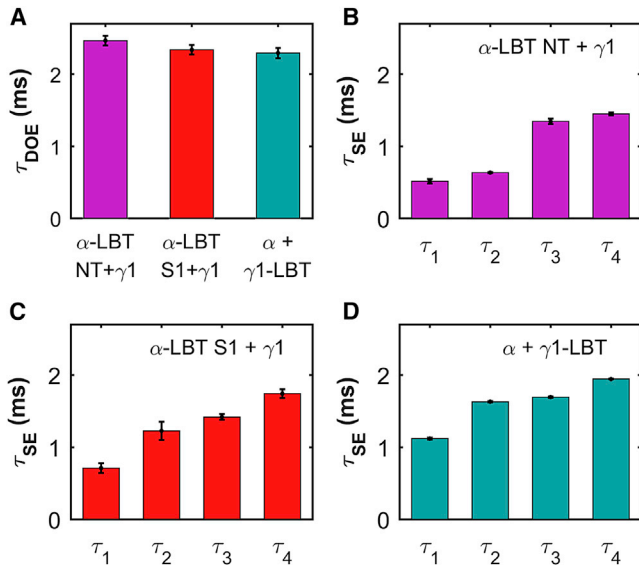


FIGURE 2 Time constants describing DOE and SE. (A) Largest time constant is shown that was obtained from three-exponential fits to DOE traces. Number of independent DOE experiments (N) was 23, 38, and 19 for (α -LBT NT + γ 1), (α -LBT S1 + γ 1), and (α + γ 1-LBT)BK channels, respectively. (B–D) The four largest time constants are shown that were obtained from Heyduk-constrained (23) SNPS fit from SE traces (α -LBT NT + γ 1)BK channels (B, $N = 15$), (α -LBT S1 + γ 1)BK channels (C, $N = 5$), and (α + γ 1-LBT)BK channels (D, $N = 12$). Bar height represents the mean, and the error-bar length represents the mean \pm SE (Table S2). To see this figure in color, go online.

where W_α is a factor, proportional to the number of α -subunits in the microscope visual field for each experiment; $\tau_{\alpha,i}$ values are the four time constants corresponding to each different donor-acceptor distance; $U(t)$ is the laser-induced fast transient artifact; and c is the offset. In Eq. 1, each preexponential $k_{\alpha,i}$ is related to each $\tau_{\alpha,i}$ by the Heyduk constraint (23),

$$k_{\alpha,i} = 1/\tau_{\alpha,i} - 1/\tau_{\text{DOE}}, \quad (2)$$

and the fast transient artifact was described by a two-exponential function

$$U(t) = A_{1,\text{Art}}e^{-t/\tau_{1,\text{Art}}} + A_{2,\text{Art}}e^{-t/\tau_{2,\text{Art}}}. \quad (3)$$

Similarly, (α + γ 1-LBT)BK channels SE decays (Fig. S2 C, bottom) were analyzed using the equation

$$I(t) = W_{\gamma 1} \sum_{j=1}^4 k_{\gamma 1,j} e^{-t/\tau_{\gamma 1,j}} + U(t) + c, \quad (4)$$

where $W_{\gamma 1}$ is proportional to the number of γ 1 subunits present in BK channels in the microscope visual field, and $\tau_{\gamma 1,j}$ are the four time constants corresponding to each different donor-acceptor distance.

The two time constants of the fast transient artifact were always smaller than those from SE (data not shown). From all parameters describing SE decays (Eqs. 1 and 4), only τ values (and therefore k values) are relevant and the rest are artifacts. The set of the four largest time constants obtained from the SNPS fit of several experiments for each preparation were averaged and they are shown in the Fig. 2, B–D, and Table S2.

Interestingly, the SE kinetics of (α + γ 1-LBT)BK channels (Fig. S2 C, bottom; Fig. 2 D) is slower than either of the two (α -LBT + γ 1)BK channels tested (Fig. S2, A and B, bottom; Fig. 2, B and C; Table S2), indicating that the position of the γ 1-LBT is further away from the pore than the NT and S1 segments of the α -subunit.

When both proteins, α and γ 1, contain LBTs, it is expected that the SE decay can be described by the linear combination of the first sums in Eqs. 1 and 4, plus the artifact and the offset. Adding Eqs. 1 and 4 and rearranging, we have

$$I(t) = W_\alpha \left[\sum_{i=1}^4 k_{\alpha,i} e^{-t/\tau_{\alpha,i}} + \frac{W_{\gamma 1}}{W_\alpha} \sum_{j=1}^4 k_{\gamma 1,j} e^{-t/\tau_{\gamma 1,j}} \right] + U(t) + c. \quad (5)$$

In Eq. 5, $W_{\gamma 1}/W_\alpha$ is the stoichiometric ratio between the γ 1- and α -subunits. To obtain the SE originated by coexpressing α -LBT and γ 1-LBT constructs, *Xenopus laevis* oocytes were coinjected with a mix of mRNA for each one of the α -LBT constructs with γ 1-LBT. One representative record of SE obtained from (α -LBT NT + γ 1-LBT)BK channels is shown in Fig. 3 A. After the offset subtraction using decay analysis (16), there are only six free parameters in Eq. 5 because both time constants and amplitudes of the exponentials contained in the sums were obtained from experiments in which the SE produced by the pairs (α -LBT + γ 1) and (α + γ 1-LBT) was fitted using Eqs. 1 and 4. The record shown in Fig. 3 A was fitted using a custom script using the MATLAB function *lsqnonlin* (the MathWorks, Natick, MA), and the weighted residuals are shown in Fig. 3 B. We started the curve fitting from 100 sets of the six initial adjustable parameters randomly chosen within reasonable limits. This strategy allowed us to minimize the probability that the obtained solution was a local minimum of the sum of the square of the weighted residuals. We found that all 100 fits converged to the same set of final parameters (Fig. 3 C). In the particular case of Fig. 3 A, the $W_{\gamma 1}/W_\alpha$ ratio was ~ 1 (Fig. 3 C, right).

The mRNA for each α -LBT construct and γ 1-LBT was coexpressed in different concentration ratios, and their SEs recorded as in Fig. 3 were fitted using Eq. 5. In both cases, the mean value of $W_{\gamma 1}/W_\alpha$ ratio increases approaching an asymptotic value of ~ 1 when the amount of mRNA of γ 1-LBT was saturating (Fig. 4, A and B). From results in Fig. 4, we concluded that the maximal

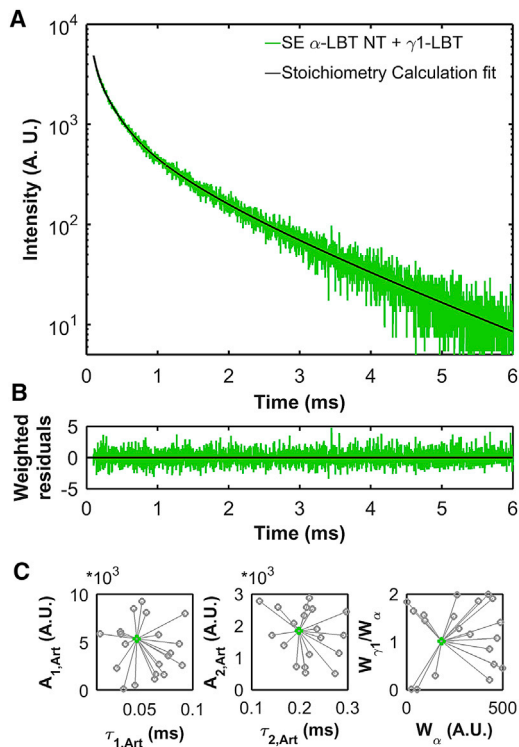


FIGURE 3 Stoichiometry determination. (A) SE measurements are given from the α -LBT NT in the presence of $\gamma 1$ -LBT. The SE was fitted using Eq. 5 (black line). The $[\text{mRNA}_{\gamma 1\text{-LBT}}]/[\text{mRNA}_{\alpha\text{-LBT NT}}]$ ratio was 3:1. The voltage was clamped at -80 mV using the two-electrodes voltage-clamp technique. The SE trace was offset-subtracted. (B) Weighted residuals are given between SE trace and its fit using Eq. 5. (C) One-hundred sets of the six-initial adjustable parameters were randomly generated within reasonably chosen boundaries for the fit of the six parameters from Eq. 5. Pairs of the values of 20 of the 100 sets are shown as open circles ($A_{1,\text{Art}}$ versus $\tau_{1,\text{Art}}$, left; $A_{2,\text{Art}}$ versus $\tau_{2,\text{Art}}$, middle; and $W_{\gamma 1}/W_{\alpha}$ versus W_{α} , right). In the 100 fits, all solutions converged to the same value (filled circle). The starting pairs of values and their final solutions are connected by a line. To see this figure in color, go online.

stoichiometry of association between the α - and the $\gamma 1$ subunits of BK channel is 1:1, whereby there are four $\gamma 1$ subunits per BK channel. This is similar to the maximal stoichiometry of the $(\alpha + \beta 1)$ BK channel complex (14).

We recall here that $\gamma 1$ (wt) and $\gamma 1$ -LBT produce only a modest leftward shift of the G-V curve when they are coexpressed with α -LBT S1, but they produce the full effect when they are coexpressed with α (wt) (Fig. 1). Conversely, α -LBT NT in the presence of $\gamma 1$ shows a behavior almost identical to the wt α -subunit (Fig. 1). These results suggest that S1, or at least part of this transmembrane segment, participates in the physiological $\gamma 1$ effect but not in the binding because the α -LBT S1 construct retains the same maximal association stoichiometry as α -LBT NT (Fig. 4, A and B).

Some questions remain to be resolved. The results shown in Fig. 4 cannot answer whether the α -homotetramer can hold fewer than four $\gamma 1$ subunits, because the $W_{\gamma 1}/W_{\alpha}$ ratio

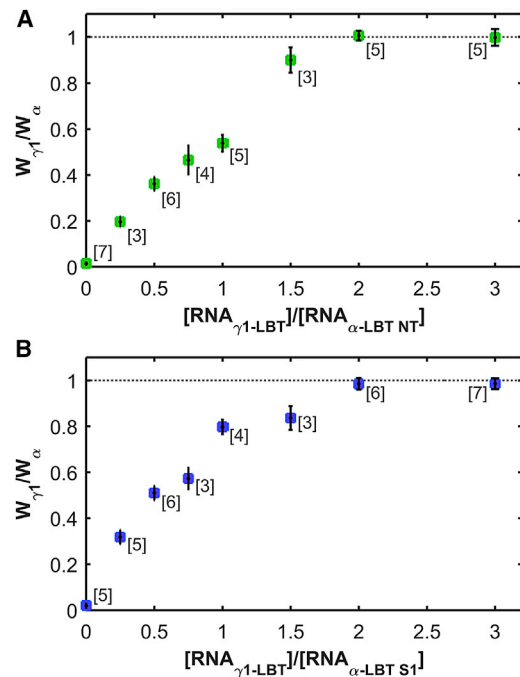


FIGURE 4 Stoichiometry of the $(\alpha + \gamma 1)$ BK channel complex. (A) Several $[\text{mRNA}_{\gamma 1\text{-LBT}}]/[\text{mRNA}_{\alpha\text{-LBT NT}}]$ and (B) $[\text{mRNA}_{\gamma 1\text{-LBT}}]/[\text{mRNA}_{\alpha\text{-LBT S1}}]$ ratios were injected into *Xenopus laevis* oocytes and the SE lifetime measurements were fitted with Eq. 5, as described in Fig. 3. In both cases, the stoichiometric ratio tends to an asymptotic value of 1. At each ratio, circles and error bars represent the mean and the mean \pm SE, respectively. The number of experiments for each ratio is indicated between square brackets. To see this figure in color, go online.

as a function of the $[\text{RNA}_{\gamma 1\text{-LBT}}]/[\text{RNA}_{\alpha\text{-LBT}}]$ ratio only tell us the total proportion of $\gamma 1$ -LBT subunits in relation to the α -LBT. Gonzalez-Perez et al. (15) found that the $\gamma 1$ effect is all-or-none, but they did not find conclusive evidence on what is $\gamma 1:\alpha$ stoichiometry between both proteins. Thus, a more intriguing question is how many $\gamma 1$ subunits are needed to cause the all-or-none $\gamma 1$ effect (15). Also, taking into account the results shown in this letter, it would be interesting to discriminate between two possibilities when there are nonsaturating $\gamma 1$ expression levels. BK channels could display partial stoichiometries (from 0 to 4 $\gamma 1$ subunits per channel) or on the contrary, could be expressed as a mix of K channels containing 0 or 4 $\gamma 1$ subunits without intermediate stoichiometries.

SUPPORTING MATERIAL

Supporting Materials and Methods, two figures, and two tables are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(18\)30448-X](http://www.biophysj.org/biophysj/supplemental/S0006-3495(18)30448-X).

AUTHOR CONTRIBUTIONS

R.L., F.B., O.A., and W.C.-U. designed the research, analyzed data, and wrote the manuscript. W.C.-U. performed research. F.B. contributed analytic tools.

ACKNOWLEDGMENTS

We thank Mrs. Luisa Soto and Mrs. Victoria Prado (University of Valparaiso) for technical assistance.

This research was supported by The National Fund for Scientific and Technological Development (FONDECYT) grant 1150273 and the US Air Force Office of Scientific Research (AFOSR) under award No. FA9550-16-1-0384 to R.L.; Anillo Grant ACT-1107; and National Institutes of Health grants GM030376 and U54GM087519 to F.B. The Centro Interdisciplinario de Neurociencia de Valparaiso is a Millennium Institute supported by the Millennium Scientific Initiative of the Chilean Ministry of Economy, Development, and Tourism (P029-022-F).

SUPPORTING CITATIONS

Reference (24) appears in the [Supporting Material](#).

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Supplemental Information

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Supporting Material

Determination of the stoichiometry between α and $\gamma 1$ subunits of the BK channel using LRET

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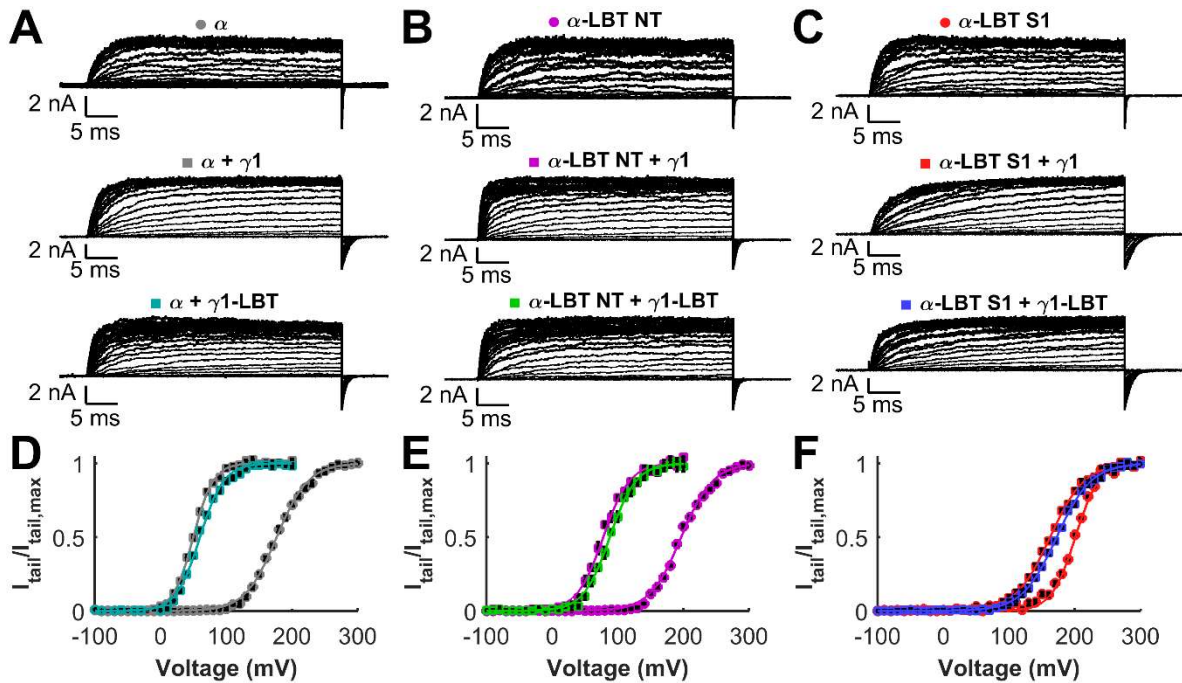


FIGURE S1. Biophysical characterization of LBT constructs.

(A-C) Representative records of BK channel macroscopic currents evoked by 40 ms voltage pulses (-100 to 200 or 300 mV; in 10 mV steps). BK pore was formed by the wild-type (wt) α subunit (A) and two different LBT-labeled α constructs, NT (B) and S1 (C). α (wt) and α -LBT constructs, were expressed in three different experimental conditions: α subunit alone (top); coexpressed with $\gamma 1$ (wt) subunit (middle); and, coexpressed with $\gamma 1$ -LBT subunit (bottom). (D-F) Normalized tail currents versus voltage [$(I_{tail}/I_{tail,max})$ -V] curves obtained from experiments like those in A-C. Data were fitted using Boltzmann function (solid lines). (D) For BK channels whose pore was formed by the α (wt) subunit, fitted parameters were (mean \pm S.E.M.): $V_h=174\pm 12$ mV and $z\delta=1.06\pm 0.17 e_0$, for α BK channels (N=9); $V_h=55\pm 4$ mV and $z\delta=1.72\pm 0.04 e_0$, for ($\alpha + \gamma 1$)BK channels (N=12); $V_h=60\pm 3$ mV and $z\delta=1.46\pm 0.07 e_0$, for ($\alpha + \gamma 1$ -LBT)BK channels (N=13). (E) For BK channels whose pore was formed by the α -LBT NT construct, fitted parameters were (mean \pm S.E.M.): $V_h=195\pm 7$ mV and $z\delta=1.08\pm 0.08 e_0$, for (α -LBT NT)BK channels (N=4); $V_h=76\pm 5$ mV and $z\delta=1.33\pm 0.10 e_0$, for (α -LBT NT + $\gamma 1$)BK channels (N=6); $V_h=82\pm 7$ mV and $z\delta=1.35\pm 0.12 e_0$, for (α -LBT NT + $\gamma 1$ -LBT)BK channels (N=4). (F) For BK channels whose pore was formed by the α -LBT S1 construct, fitted parameters were (mean \pm S.E.M.): $V_h=200\pm 3$ mV and $z\delta=1.42\pm 0.16 e_0$, for (α -LBT S1)BK channels (N=4); $V_h=159\pm 10$ mV and $z\delta=1.01\pm 0.08 e_0$, for (α -LBT S1 + $\gamma 1$)BK channels (N=9); $V_h=165\pm 12$ mV and $z\delta=1.01\pm 0.09 e_0$, for (α -LBT S1 + $\gamma 1$ -LBT)BK channels (N=5).

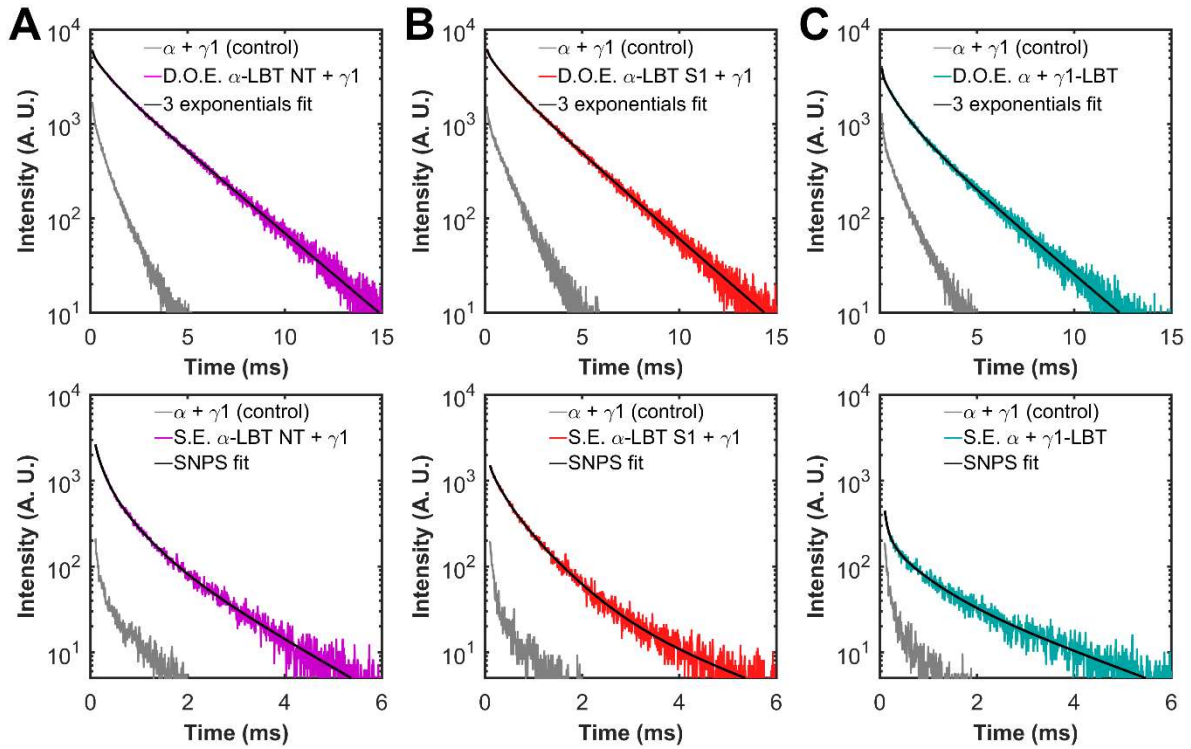


FIGURE S2. LRET measurements.

(A-C) Donor only emission (DOE; [top]) and sensitized emission (SE; [bottom]) lifetime measurements from the α -LBT NT (A) and S1 (B) constructs in the presence of $\gamma 1$ (wt) (purple and red, respectively) and from the α (wt) in the presence of $\gamma 1$ -LBT (C; cyan). Three exponentials and SNPS fit (1) (black) are shown for DOE and SE traces, respectively. A control/background trace from ($\alpha + \gamma 1$)BK channels is also shown in each panel (gray). Experiments were performed while the voltage was clamped at -80 mV with the two-microelectrode voltage clamp technique. All traces were offset-subtracted. Note different time scales in DOE traces versus SE traces.

TABLE S1. Donor only time constants. Mean \pm S.E.M.

BK channel composition	τ_{DOE} (ms)	N
α-LBT NT + γ1	2.46 ± 0.07	23
α-LBT S1 + γ1	2.34 ± 0.07	38
α + γ1-LBT	2.29 ± 0.07	19

TABLE S2. Sensitized emission time constants. Mean \pm S.E.M.

BK channel composition	τ_1 (ms)	τ_2 (ms)	τ_3 (ms)	τ_4 (ms)	N
α-LBT NT + γ1	0.51 \pm 0.03	0.63 \pm 0.01	1.34 \pm 0.04	1.45 \pm 0.02	15
α-LBT S1 + γ1	0.71 \pm 0.07	1.23 \pm 0.12	1.42 \pm 0.04	1.74 \pm 0.06	5
α + γ1-LBT	1.12 \pm 0.01	1.63 \pm 0.01	1.69 \pm 0.01	1.95 \pm 0.01	12

METHODS

Mutagenesis. The cDNA coding for the human BK α -subunit (*KCNMA1*) from myometrium was provided by Dr. Ligia Toro (University of California, Los Angeles, CA). The cDNA coding for the human $\gamma 1$ subunit (*LRRC26*) was kindly provided by Dr. Jiusheng Yan (University of Texas MD Anderson Cancer Center, Houston, TX) and Dr. Richard Aldrich (University of Texas, Austin, TX). The DNA sequence encoding LBT (YWDTNNDGWYEGDELLA (2)) was inserted in chosen positions of the α and $\gamma 1$ subunits using standard molecular biology techniques. Insertions were confirmed by sequencing.

LRET acceptor. The LRET acceptor was the fluorophore BODIPY attached to the scorpion toxin iberiotoxin (IbTX). Labeled [D19C]IbTX was synthesized as previously described (3).

Heterologous protein expression. The wild-type and mutants cDNA were transcribed using T7 polymerase transcription kit (Ambion). The mRNA solution (50 nl) was injected into *Xenopus laevis* oocytes. For all experiments, α and α -LBT constructs RNA concentration were 250 ng/ μ l. In (α -LBT + $\gamma 1$)BK experiments, $\gamma 1$ mRNA concentration was 750 ng/ μ l (ratio $[RNA_{\gamma 1}]/[RNA_{\alpha-LBT}]=3$). In (α -LBT + $\gamma 1$ -LBT)BK experiments, $\gamma 1$ -LBT mRNA concentration tested were 500, 375, 250, 187.5, 125, and 62.5 ng/ μ l (ratios $[RNA_{\gamma 1-LBT}]/[RNA_{\alpha-LBT}]=2, 1.5, 1, 0.75, 0.5, 0.25$, respectively). In the last cases, the mix solutions were complemented with mRNA for $\gamma 1$ wt at 0, 125, 250, 312.5, 375 and 437.5 ng/ μ l to assure that the total $\gamma 1$ mRNA was constant and in excess along the experiments. An additional $[RNA_{\gamma 1-LBT}]/[RNA_{\alpha-LBT}]$ ratio of 3 ($[RNA_{\gamma 1-LBT}]=750$ ng/ μ l; $[RNA_{\alpha-LBT}]=250$ ng/ μ l) was tested. Oocytes were incubated in ND96 (96 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.6) solution at 18 °C for 3–5 days before recording.

Electrophysiology and LRET experiments. The electrophysiological characterization was made under patch clamp with an Axopatch 200B amplifier in the inside-out configuration. The bath and pipette solution both contained (in mM) K-MES 108, KCl 2, HEPES 10, and EGTA 5 (pH 7.4). The solution contained an estimated 0.8 nM Ca²⁺, assuming the presence of 10 μ M Ca²⁺ contaminant (4). During LRET experiments, the voltage was held to -80 mV using a CA-1B amplifier (Dagan Corp.) in the two-electrode voltage-clamp configuration. The internal resistance of the microelectrodes was in the 0.4–0.8 M Ω range and the pipette solution was 3 M KCl. During donor only emission (DOE) experiments, the bath solution contained NMDG-MES 110 mM HEPES 10 mM, CaCl₂ 2 mM, and TbCl₃ 10 μ M (pH 7.4). For sensitized emission (SE) experiments, BODIPY-[D19C]IbTX stock solution was added to the bath solution until reaching 500 nM concentration and the oocytes were incubated for 5 min before recording.

Optical Setup. The optical setup used for simultaneous LRET and electrophysiology experiments was custom-designed as has been previously described (1,3). DOE and SE traces were obtained by averaging 16–25 pulses.

Donor only emission analysis. The analysis of the DOE decays was performed using the Decay Analysis software (1). The first 15 ms from each DOE trace was fitted to a three-exponential decay plus offset. The largest time constant (>2.25 ms) was considered as the decay time constant of Tb³⁺ bound to LBT (τ_{DOE}).

Sensitized emission analysis. The analysis of the SE decays was performed using the SNPS software (1). The position of the acceptor was the reported by (3). The first 6 ms from each SE trace was fitted to a Heyduk-constrained (5) tetra-exponential decay plus a two-exponential artifact plus offset. From

the output of the SNPS program the four time constants of the best fit to the $(\alpha\text{-LBT} + \gamma\text{1})\text{BK}$ and $(\alpha + \gamma\text{1-LBT})\text{BK}$ SE traces were obtained.

Stoichiometry determination. The offset of $(\alpha\text{-LBT} + \gamma\text{1-LBT})\text{BK}$ SE traces was calculated and subtracted to the trace using Decay Analysis software (1). The offset-subtracted trace was fitted with the Eq. 5 using a custom script using the Matlab function *lsqnonlin*, which solve nonlinear least-squares problems. The boundaries of the values of the six parameters were reasonably chosen and the six-element vectors of start values were randomly generated. For each experiment, 100 start values vectors were generated and 100 independent fits were made, to visually inspect that the solution was not a local minimum of the sum of the square of the weighted residuals.

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