Regular Article*Highlighted Paper selected by Editor-in-Chief***Single Molecule Fluorescence Imaging Reveals the Stoichiometry of BK γ 1 Subunit in Living HEK293 Cell Expression System**

Sayuri Noda, Yoshiaki Suzuki, Hisao Yamamura, and Yuji Imaizumi*

Department of Molecular & Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University; 3-1 Tanabedori, Mizuho-ku, Nagoya 467-8603, Japan.

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Large conductance Ca^{2+} -activated K^+ (BK_{Ca}) channels are ubiquitously expressed in plasma membrane of both excitable and non-excitable cells and possess significant physiological functions. A tetrameric complex of α subunit ($\text{BK}\alpha$) forms a functional pore of BK_{Ca} channel. The properties of BK_{Ca} channel, such as voltage-dependence, Ca^{2+} sensitivity and pharmacological responses, are extensively modulated by co-expressing accessory β subunits ($\text{BK}\beta$), which can associate with $\text{BK}\alpha$ in one to one manner. Although the functional significance of newly identified γ subunits ($\text{BK}\gamma$) has been revealed, the stoichiometry between $\text{BK}\alpha$ and $\text{BK}\gamma$ 1 remains unclear. In the present study, we utilized a single molecule fluorescence imaging with a total internal reflection fluorescence (TIRF) microscope to directly count the number of green fluorescent protein (GFP)-tagged $\text{BK}\gamma$ 1 ($\text{BK}\gamma$ 1-GFP) within a single BK_{Ca} channel complex in HEK293 cell expression system. $\text{BK}\gamma$ 1-GFP significantly enhanced the BK channel activity even when the intracellular Ca^{2+} concentration was kept lower, *i.e.*, 10 nM, than the physiological resting level. $\text{BK}\gamma$ 1-GFP stably formed molecular complexes with $\text{BK}\alpha$ -mCherry in the plasma membrane. Counting of GFP bleaching steps revealed that a BK_{Ca} channel can contain up to four $\text{BK}\gamma$ 1 per channel at the maximum. These results suggest that $\text{BK}\gamma$ 1 forms a BK_{Ca} channel complex with $\text{BK}\alpha$ in a 1 : 1 stoichiometry in a human cell line.

Key words K^+ channel; single molecule imaging; patch-clamp recording; large conductance Ca^{2+} -activated K^+ channel; large conductance Ca^{2+} -activated K^+ channel gamma 1 subunit; total internal reflection fluorescence microscope

INTRODUCTION

Large-conductance Ca^{2+} -activated K^+ (BK_{Ca}) channel plays significant physiological roles in various cell types of different tissues, such as neurons, smooth muscles, secretory glands and cartilages.¹⁾ It has been well known that BK_{Ca} channel consists of pore-forming α subunit ($\text{BK}\alpha$) and auxiliary β subunit ($\text{BK}\beta$). $\text{BK}\alpha$ is encoded by a single gene, whereas four subtypes of $\text{BK}\beta$ ($\text{BK}\beta$ 1– β 4) have been reported to give tissue-specific modifications to BK channel activities by their tissue-dependent expression.²⁾ Recently leucine-rich-repeat-containing (LRR) proteins have been identified as novel additional auxiliary $\text{BK}\gamma$ subunits ($\text{BK}\gamma$).³⁾ LRR26, LRR52, LRR55 and LRR38 have been specifically re-named as the $\text{BK}\gamma$ 1, $\text{BK}\gamma$ 2, $\text{BK}\gamma$ 3 and $\text{BK}\gamma$ 4 for BK_{Ca} channels, respectively.⁴⁾ The incorporation of $\text{BK}\gamma$ to BK_{Ca} channel complex promotes a voltage-shift of channel activation to very negative membrane potentials, even without an increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{i}}$).^{3,5)} In addition, $\text{BK}\gamma$ 1 can also modulate pharmacological features of BK_{Ca} channels.⁶⁾ At present, $\text{BK}\gamma$ 1 has been known to function as a BK_{Ca} channel auxiliary subunit in parotid acinar cells,^{6,7)} airway epithelial cells^{8,9)} and bronchial and vascular smooth muscle cells (SMCs).^{5,10)}

It has been also reported that $\text{BK}\gamma$ regulates BK_{Ca} channel functions in an all-or-none manner.¹¹⁾ This implies that only one $\text{BK}\gamma$ interacts with $\text{BK}\alpha$ tetramer can fully activate the BK_{Ca} channel activity. In contrast, two independent studies recently suggested that a BK_{Ca} channel can incorporate up to four $\text{BK}\gamma$ 1 using fluorescence resonance energy transfer

(FRET) analysis and single channel recordings¹²⁾ or lanthanide resonance energy transfer.¹³⁾ However, these studies do not directly count the number of $\text{BK}\gamma$ 1 within a single functional BK_{Ca} channel. In addition, they used *Xenopus* oocytes that have different protein expression system from mammalian cells.¹⁴⁾ In the present study we directly counted $\text{BK}\gamma$ 1 within a single functional BK_{Ca} channel expressed in a human-derived cell line, HEK293 cells, using single molecular imaging with a total internal reflection fluorescence (TIRF) microscope.

MATERIALS AND METHODS

Cell Culture The HEK293 cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan). Cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 U/mL penicillin, and 20 $\mu\text{g}/\text{mL}$ streptomycin (Sigma-Aldrich, U.S.A.) at 37°C.

TIRF Imaging Single molecule imaging was performed using a TIRF imaging system with an objective lens (CFI Apo TIRF 100 $\times$ /1.49, oil immersion; Nikon, Tokyo, Japan) as described previously.^{15,16)} Data were collected with an EM-CCD camera and analyzed by AQUACOSMOS software (Hamamatsu Photonics, Hamamatsu, Japan). Green fluorescent protein (GFP)- or mCherry-fused protein targets were excited with a 488-nm argon laser (Spectra-Physics, Santa Clara, CA, U.S.A.) or a 543-nm He/Ne laser (Melles Griot, Carlsbad, CA, U.S.A.), respectively. GFP/mCherry emission data were collected using a combination of dichroic mirrors and dual band-pass filters (505–530/570–660 nm; Omega Optical, Brat-

* To whom correspondence should be addressed. e-mail: yimaizumi@phar.nagoya-cu.ac.jp

tleboro, VT, U.S.A.). The resolution of images was 105 nm per pixel (x - y) and 200 nm (z). All experiments were carried out at room temperature (25°C).

Plasmid Constructs and Transfection Full-length of cDNA encoding the human KCNMA1 (BK α , NM_002247) was subcloned into mCherry-N1 (BK α -mCherry) (Clontech Laboratories, Mountain View, CA, U.S.A.). Human KCNB1 (BK β 1, NM_004137) or LRRC26 (BK γ 1, NM_001013653) were also subcloned into pAcGFP-N1 (BK β 1-GFP and BK γ 1-GFP). The fusion construct in which N-terminus of BK γ 1-GFP is fused to the C-terminus of BK α -mCherry (BK α + BK γ 1) was also established to realize equal expression of BK α and BK γ 1 within a HEK293 cell.³⁾ In this construct, BK γ 1-GFP is cleaved at N-terminal signal peptide sequences, separated from BK α -mCherry at ER and exported to plasma membrane.³⁾ All constructs were confirmed by DNA sequencing. HEK293 cells were transiently transfected with cDNA (each 1 μ g for co-expression) using LipofectAMINE 2000 (Invitrogen, Waltham, CA, U.S.A.). Experiments were performed 24 to 48 h after transfection. It has been confirmed that the functions or properties of these constructs tagged with fluorescent proteins are equivalent to those of native constructs.^{3,5,15)}

Electrophysiological Recordings Electrophysiological studies were performed using conventional whole-cell voltage-clamp and current-clamp techniques with a CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan), an analog-digital converter (DIGIDATA 1440A; Molecular Devices, Sunnyvale, CA, U.S.A.) and a pCLAMP software (version 10.2; Molecular Devices), as described previously.¹⁶⁾ For measurements of whole-cell BK Ca channel currents, the pipette filling solution contained (mM): 140 KCl, 0.265 CaCl₂, 4 MgCl₂, 10 *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), 2 Na₂ATP and 5 ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) (pCa8.0). The pH was adjusted to 7.2 with 1 M KOH. The extracellular solution had an ionic composition of (mM) 137 NaCl, 5.9 KCl, 2.2 CaCl₂, 1.2 MgCl₂, 14 glucose and 10 HEPES. The pH was adjusted to 7.4 with NaOH. BK Ca channel currents were activated from a holding potential of -80 mV by applying 80-ms voltage steps to voltages between -100 and +100 mV in increments of 10 mV. All experiments were carried out at room temperature (25°C). The total cell membrane capacitance was measured to confirm single cell recording and for calculation of current density per capacitance of unit membrane area (BK α , 27.2 ± 1.0 pF, $n = 4$; BK α + γ 1, 20.4 ± 1.9 pF, $n = 4$).

Single Molecule GFP Bleaching The number of BK γ 1 subunits in the immediate vicinity of the plasma membrane of HEK293 cells was counted by observing the bleaching steps of GFP fused to the subunits in a single particle, as described previously.^{16,17)} Images were obtained in the order of mCherry and GFP to avoid bleaching by cross-excitation. Images were acquired at 10 Hz for 100–120 s. Fluorescence intensity in a region of interest (ROI) (5 $\times$ 5 pixels) was calculated by subtracting the background in 24 pixels around the ROI. The number of bleaching steps was determined by eye from the fluorescence signal trace. The distributions of observed number of bleaching steps were compared with zero-truncated binomial distribution, $P(x, n, p) = {}_nC_x p^x (1-p)^{n-x}$, where n is the number of subunits ($n = 4$ for tetramers), x is the observed number of bleaching steps, and p is the apparent probability for GFP fluorescence.¹⁸⁾

Reagents Paxilline (Alomone Laboratories) was dissolved in dimethyl sulfoxide (DMSO) as a 1 mM stock solution. The final concentration of DMSO was 0.1%. Stock solutions were stored -20°C prior to use.

Statistics Pooled data are presented as the mean $\pm$ standard error of the mean (S.E.M.) Statistical significance between two groups was evaluated using *t*-test after *F*-test application. When the distribution between the two groups was equal, Student's *t*-test was used. If not, Welch's *t*-test was utilized for statistics analysis. $p < 0.05$ was considered statistically significant.

RESULTS

BK Ca Channel Currents of HEK 293 Cells Expressing BK α and BK γ 1 When BK α labeled with mCherry or BK α + BK γ 1 labeled with GFP were transiently expressed in HEK293 cells, outward currents were recorded upon depolarization under voltage clamp (Fig. 1A). BK α labeled with mCherry shows a typical BK channel single channel conductance (approximately 230 pS) and it was equivalent to that of non-labeled BK α .¹⁵⁾ [Ca²⁺]_i in the pipette solution was kept

Fig. 1. BK Ca Channel Currents of HEK 293 Cells Expressing BK α and BK γ 1

(A) Typical traces of outward currents from HEK293 cells expressing BK α or BK α + BK γ 1 before (control) and after the treatment with 1 μ M paxilline, a selective BK Ca channel blocker. BK α and BK γ 1 were labeled with mCherry and GFP, respectively. Holding potential was -80 mV. Voltage steps from -100 to +100 mV in 10 mV increments were given to cells. The Ca²⁺ concentration in the pipette filling solution was fixed at pCa8.0. (B) The relationship between the density of paxilline-sensitive current and activation voltage in BK α ($n = 4$) and BK α + BK γ 1 ($n = 4$) is shown. The current density was obtained by dividing the amplitude of peak outward current with cell membrane capacitance. (C) The paxilline-sensitive current density at +50 mV in BK α ($n = 4$) and BK α + BK γ 1 ($n = 4$) is compared.

low at pCa 8.0 (*i.e.*, 10nM). The depolarization-induced currents were inhibited by a BK_{Ca} channel blocker, 1 μ M paxilline. In the BK α expressing cells, 1 μ M paxilline-sensitive outward currents were detected only at much higher voltages than that in BK α + BK γ 1 (Figs. 1A, B). As shown in Fig. 1C, the paxilline-sensitive current density in BK α + BK γ 1 at +50mV was markedly larger than that in BK α (BK α : 1.3 ± 2.2 pA/pF; $n = 4$, BK α + BK γ 1: 93.4 ± 23.4 pA/pF; $n = 4$, $p = 0.0294$ vs. BK α). These results showed that BK α and BK γ 1 labeled with mCherry and GFP possess function to elicit paxilline-sensitive and voltage-dependent outward currents and that co-expression of BK γ 1 with BK α extensively enhanced the current amplitude under the experimental conditions ($[Ca^{2+}]_i = 10$ nM). Importantly, BK γ 1 labeled with GFP enhanced BK_{Ca} channel activity to the same extent as non-labeled BK γ 1 does.^{3,5)}

BK γ 1 Stably Forms Molecular Complexes with BK α in the Plasma Membrane The stability of BK α -BK γ 1 complex and also the effect of molecular complex formation on BK α mobility in the plasma membrane were measured by visualizing BK α -mCherry and BK γ 1-GFP in live cells using single molecular imaging system with a TIRF microscope (Fig. 2A).

Fig. 2. The Mobility of BK α Was Decreased by Interaction with BK γ 1

The localization and behavior of BK α and BK γ 1 in plasma membrane were visualized by using a TIRF microscope. BK α alone or BK α + BK γ 1 (BK α -mCherry and BK γ 1-GFP) was transiently expressed in HEK293 cells. (A) Typical TIRF image of HEK293 cells expressing BK α + BK γ 1. (B) The particles of BK α -mCherry and BK γ 1-GFP on the membrane surface, indicated by the image in A (particle a and b), were tracked for 60 s. (C) Molecular behavior of BK α colocalized with BK γ 1 particle was imaged every 5 s. Note that BK α and BK γ 1 moved around together in a very limited area during the recording for over 60 s. (D) Diffusion coefficient (D_{60s}) values of BK α interacting with BK γ 1 (BK α + BK γ 1) ($n = 10$) or BK α alone ($n = 20$) are shown.²³⁾ (Color figure can be accessed in the online version.)

When BK α + BK γ 1 was expressed in HEK293 cells, these proteins can be visualized as dot-like fluorescent signals. BK α -mCherry alone showed dynamic mobility on the membrane surface in HEK293 cells (Fig. 2B). As shown in Figs. 2B and C, some BK α -mCherry particles got colocalized with BK γ 1-GFP on the membrane surface. During the measurements for 60 s, two particles kept moving together on the plasma membrane. Furthermore, the mobility of BK α -mCherry was significantly decreased by the colocalization with BK γ 1-GFP (distribution efficiency (D_{60s} , $\mu m^2/s$): BK α -mCherry alone 0.052 ± 0.013 , $n = 20$; BK α -mCherry colocalized with BK γ 1-GFP 0.010 ± 0.006 , $n = 10$, $p = 0.0406$ vs. BK α -mCherry alone, Fig. 2D). These data directly demonstrate that BK γ 1 form a stable complex with BK α and BK γ 1 markedly stabilizes BK α movement in the plasma membrane of living cells.

BK γ 1 Form BK_{Ca} Channel Complex with BK α in a 1:1 Stoichiometry in Native Mammalian Cells The stoichiometry between BK α and BK γ 1 within a functional BK_{Ca} channel

Fig. 3. Four BK γ 1 Can Be Incorporated in a Single BK_{Ca} Channel Complex

BK α + BK γ 1 or BK α -mCherry and BK γ 1-GFP (transfection ratio was 1:1) were transiently expressed in HEK293 cells. Based on GFP bleaching method, the bleaching steps of GFP signals in a TIRF region were counted for determining the number of BK β 1 or BK γ 1 within a single fluorescent particle. (A) Typical TIRF images of HEK293 cells expressing BK α + BK γ 1 are shown. Analyzed particles are indicated by white circles. Only GFP particles colocalized with mCherry particles were analyzed. (B, C) Changes in GFP fluorescence intensity of BK β 1-GFP (B) and BK γ 1-GFP (C) are presented. Solid lines indicate bleaching step(s). Dotted line indicates complete bleaching (basal) level. (D) The number of bleaching steps of BK β 1-GFP and BK γ 1-GFP were summarized. The blue bars indicate the binominal distribution expected for tetramers with probability of 0.75 for a single GFP to be fluorescent. (Color figure can be accessed in the online version.)

complex was studied using a single molecule GFP bleaching method^{16,17)} (Fig. 3A). In this method, bleaching steps of GFP signal was counted in a visualized auxiliary subunit (BK γ 1 or BK β 1), that was colocalized with mCherry-labeled BK α detected as a merged fluorescent signal. It has been shown that one to four BK β 1 can bind to a BK α tetramer and increase the channel activity depending on its stoichiometry.¹⁹⁾ Therefore, we used BK β 1 as a control in this experiment.

First, HEK293 cells co-expressing BK α -mCherry and BK β 1-GFP were prepared by transfection of cDNA at a ratio of 1:1. The colocalization ratio of BK α -mCherry and BK β 1-GFP was 86% (BK α alone: $14.2 \pm 4.5\%$ and BK α colocalized with BK β 1: $85.8 \pm 4.5\%$, 119 particles from 12 cells). Single spots of BK β 1-GFP colocalized with BK α -mCherry displayed mainly 3 or 4 step bleaching (1 step, 14.5%; 2 steps, 20.3%; 3 steps, 37.5%; 4 steps, 25%, 53 particles from 12 cells, Figs. 3B, D). The population distribution of the data obtained from this bleaching step analysis was well fitted by the theoretical binomial distribution for a tetramer ($n=4$), with the apparent probability of GFP being fluorescent during excitation (p) set at 0.75¹⁸⁾ (see "Materials and Methods"). This result provided evidence suggesting that each GFP spot contained four BK β 1 under these experimental conditions.

Second, HEK293 cells co-expressing BK α -mCherry and BK γ 1-GFP were prepared by the transfection with BK α + BK γ 1 plasmid. The colocalization ratio of BK α -mCherry and BK γ 1-GFP was 81% (BK α alone: $19.1 \pm 4.5\%$ and BK α colocalized with γ 1: $80.9 \pm 4.5\%$, 194 particles from 7 cells). Similarly, the BK γ 1-GFP showed a distribution that peaked at 3 or 4 steps (1 step, 10.5%; 2 steps, 15.8%; 3 steps, 47.4%; 4 steps, 26.3%, 43 particles from 7 cells) (Figs. 3C, D). This distribution was also well fitted by binomial distribution, given tetramer ($n=4$) and apparent fluorescence probability of $p=0.75$. Taken together, BK γ 1 can form tetramers with BK α at the stoichiometry of 1:1 in a similar manner as BK β 1.

DISCUSSION

So far, two groups have suggested that a tetrameric BK α complex can interact with up to four BK γ 1 using imaging and functional analyses.¹²⁾ However, they did not directly count the number of BK γ 1 within a single functional BK Ca channel. Furthermore, their data largely rely on results obtained from oocytes whose biochemical machinery does not always assemble mammalian proteins faithfully. In the present study, we directly counted BK γ 1 within a single functional BK Ca channel expressed in a human-derived cell line, HEK293 cells, using a TIRF microscope. Our data demonstrate that BK γ 1 can stably interact with BK α at the stoichiometry of 1:1 within a single BK Ca channel complex in HEK293 cells.

We previously reported that the mobility of BK α was markedly reduced by forming complexes with BK β 1.¹⁷⁾ Accordingly, we examined here whether BK γ 1 also influences the mobility of BK α on the membrane surface. As shown in Fig. 2, BK α mobility was significantly reduced by forming complex with BK γ 1 (Fig. 2). These results suggest that BK γ 1 molecule interacts with scaffold proteins *via* intracellular C-terminal, while the molecular entity of the scaffold proteins was not further examined in this study. As for BK β 1, it dynamically changes its intracellular localization in response to intracellular signaling of Ca²⁺²⁰⁾ or NO²¹⁾ in SMCs and regu-

lates BK Ca channel activity. The movement of BK γ 1 between intracellular compartment and plasma membrane with respect to its influence on BK Ca channel activity is an emergent topic and remains to be examined.

As shown in Fig. 3D, the distribution pattern of numbers of BK β 1 and BK γ 1 was well fitted by binomial distribution under the condition where "n" and apparent fluorescence probability p are 4 and 0.75, respectively. Given that fluorescence probability of GFP and colocalization ratio (86 and 81% in BK α + BK β 1 and BK α + BK γ 1 expressing cells, respectively) are equal in these two types of cells and the expression efficacy was comparable and not changed by co-expression, the binding affinity between BK γ 1 and BK α is supposed to be comparable with that between BK β 1 and BK α . It has been suggested that a BK Ca channel can contain zero to four BK β or BK γ per channel. The incorporation of one to four BK β shows incremental effects on BK Ca channel gating.¹⁹⁾ In contrast, it has been suggested that BK γ 1 affects BK Ca channel gating in an all-or-none manner and that a single BK γ 1 incorporation is enough to induce the full channel activation. However, the researchers used BK β 2- γ 1 chimeric construct but not the native γ 1 subunit.¹¹⁾ The present study, in which equal amount of cDNA of pore and auxiliary subunits are transfected in the expression system, showed that four BK β 1 or BK γ 1 can be the most preferentially incorporated in each BK Ca channel complex.

BK β 1 is thought to be a major BK Ca channel subunit in smooth muscle tissues, such as vascular²²⁾ and bronchial²³⁾ smooth muscles. Recently we demonstrated that BK γ 1 expression is exceptionally high in mouse bronchial SMCs in comparison with other several types of SMCs and that the half activation voltage ($V_{1/2}$) of BK Ca channel current is close to that in BK α + BK γ 1 in HEK293 reconstituted system.⁵⁾ The physiological impact of the finding is that BK Ca channel activity extensively contributes to keep the stable resting membrane potential at well negative to -60 mV in bronchial SMCs. Moreover, we found that the additional co-expression of BK β 1 to BK α + BK γ 1 in HEK293 cells (BK α + BK β 1 + BK γ 1) unexpectedly resulted in a slight shift of $V_{1/2}$ to positive direction. Considering that both BK β 1 and BK γ 1 are expressed in mouse bronchial SMCs, it is possible that both subunits simultaneously bind to the same BK α tetramer. In the previous and present studies, however, it was not quantitatively examined how BK β 1 and BK γ 1 can be incorporated together in a single BK Ca channel. Thus, the hot issue about the relationship between BK β and BK γ , *i.e.*, how these subunits influence the binding to BK α each other, remains to be further determined.

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Conflict of Interest The authors declare no conflict of interest.

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