**Regular Article**

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**Single Molecule Fluorescence Imaging Reveals the Stoichiometry of BKγ1 Subunit in Living HEK293 Cell Expression System**

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**INTRODUCTION**

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 (BKα) 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 coexpressing accessory β subunits (BKβ), which can associate with BKα in one to one manner. Although the functional significance of newly identified γ subunits (BKγ) has been revealed, the stoichiometry between BKz and BKγ1 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 BKγ1 (BKγ1-GFP) within a single BK\(_{ca}\) channel complex in HEK293 cell expression system. BKγ1-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. BKγ1-GFP stably formed molecular complexes with BKα-mCherry in the plasma membrane. Counting of GFP bleaching steps revealed that a BK\(_{ca}\) channel can contain up to four BKγ1 per channel at the maximum. These results suggest that BKγ1 forms a BK\(_{ca}\) channel complex with BKα 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

**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 µg/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 ×/1.49, oil immersion; Nikon, Tokyo, Japan) as described previously. 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...
tleo, 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 (BKα, NM_002247) was subcloned into mCherry-N1 (BKα-mCherry) (Clontech Laboratories, Mountain View, CA, U.S.A.). Human KCNMB1 (BKγ, NM_004137) or LRRC26 (BKγ1, NM_001013653) were also subcloned into pACGFP-N1 (BKβ-mCherry) and BKγ1-GFP. The fusion construct in which N-terminus of BKγ1-GFP is fused to the C-terminus of BKα-mCherry (BKα + BKγ1) was also established to realize equal expression of BKα and BKγ1 within a HEK293 cell. In this construct, BKγ1-GFP is cleaved at N-terminal signal peptide sequences, separated from BKα-mCherry at ER and exported to plasma membrane.

All constructs were confirmed by DNA sequencing. HEK293 cells were transiently transfected with cDNA (each 1 μg 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.

**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). The pipette filling solution contained (mM): 140 KCl, 0.265 CaCl2, 4 MgCl2, 10 N-(2-hydroxyethyl)piperezine-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. BKca 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 (BKα, 27.2 ± 1.0 pF, n = 4; BKα + γ1, 20.4 ± 1.9 pF, n = 4).

**Single Molecule GFP Bleaching** The number of BKγ1 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. Images were obtained in the order of mCherry of GFP fused to the subunits in a single particle, as described previously.

**Resuits**

**BKCa Channel Currents of HEK 293 Cells Expressing BKα and BKγ1** When BKα labeled with mCherry or BKα + BKγ1 labeled with GFP were transiently expressed in HEK293 cells, outward currents were recorded upon depolarization under voltage clamp (Fig. 1A). BKα labeled with mCherry shows a typical BK channel single channel conductance (approximately 230 pS) and it was equivalent to that of non-labeled BKα. [Ca2+]i in the pipette solution was kept constant at 4 μM paxilline, a selectiveBK Ca channel blocker. BKα and BKγ1 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 BKα (n = 4) and BKα + BKγ1 (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 BKα (n = 4) and BKα + BKγ1 (n = 4) is compared.
Fig. 2. The Mobility of BKα Was Decreased by Interaction with BKγ1

The localization and behavior of BKα and BKγ1 in plasma membrane were visualized by using a TIRF microscope. BKα alone or BKα + BKγ1 (BKα-mCherry and BKγ1-GFP) was transiently expressed in HEK293 cells. (A) Typical TIRF images of HEK293 cells expressing BKα + BKγ1. (B) The particles of BKα-mCherry and BKγ1-GFP on the membrane surface, indicated by the image in A (particle a and b), were tracked for 60 s. (C) Molecular behavior of BKα colocalized with BKγ1 particle was imaged every 5 s. Note that BKα and BKγ1 moved around together in a very limited area during the recording for over 60 s. (D) Diffusion coefficient ($D_{\text{obs}}$) values of BKα interacting with BKγ1 (BKα + BKγ1) ($n = 10$) or BKα alone ($n = 20$) are shown.²³ (Color figure can be accessed in the online version.)

Fig. 3. Four BKγ1 Can Be Incorporated in a Single BKca Channel Complex

BKα + BKγ1 or BKα-mCherry and BKγ1-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 BKβ1 or BKγ1 within a single fluorescent particle. (A) Typical TIRF images of HEK293 cells expressing BKα + BKγ1 were 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 BKβ1-GFP (distribution efficiency ($D_{\text{obs}}$, µm²/s): BKα-mCherry alone 0.052 ± 0.013, $n = 20$; BKα-mCherry colocalized with BKγ1-GFP 0.010 ± 0.006, $n = 10$, $p = 0.0406$ vs. BKα-mCherry alone, Fig. 2D). These data directly demonstrate that BKγ1 form a stable complex with BKα and BKγ1 markedly stabilizes BKα movement in the plasma membrane of living cells. BKγ1 Form BKca Channel Complex with BKα in a 1:1 Stoichiometry in Native Mammalian Cells  The stoichiometry between BKα and BKγ1 within a functional BKca channel was investigated by using a TIRF microscope (Fig. 2A).

When BKα + BKγ1 was expressed in HEK293 cells, these proteins can be visualized as dot-like fluorescent signals. BKα-mCherry alone showed dynamic mobility on the membrane surface in HEK293 cells (Fig. 2B). As shown in Figs. 2B and C, some BKα-mCherry particles got colocalized with BKγ1-GFP on the membrane surface. During the measurements for 60 s, two particles kept moving together on the plasma membrane. Furthermore, the mobility of BKα-mCherry was significantly decreased by the colocalization with BKγ1-GFP (distribution efficiency ($D_{\text{obs}}$, µm²/s): BKα-mCherry alone 0.052 ± 0.013, $n = 20$; BKα-mCherry colocalized with BKγ1-GFP 0.010 ± 0.006, $n = 10$, $p = 0.0406$ vs. BKα-mCherry alone, Fig. 2D). These data directly demonstrate that BKγ1 form a stable complex with BKα and BKγ1 markedly stabilizes BKα movement in the plasma membrane of living cells.

**BKγ1 Stably Forms Molecular Complexes with BKα in the Plasma Membrane** The stability of BKα-BKγ1 complex and also the effect of molecular complex formation on BKα mobility in the plasma membrane were measured by visualizing BKα-mCherry and BKγ1-GFP in live cells using single molecular imaging system with a TIRF microscope (Fig. 2A).
complex was studied using a single molecule GFP bleaching method. In this method, bleaching steps of GFP signal was counted in a visualized auxiliary subunit (BKα or BKβ/γ), that was colocalized with mCherry-labeled BKα detected as a merged fluorescent signal. It has been shown that one to four BK/β can bind to a BKα tetramer and increase the channel activity depending on its stoichiometry. Therefore, we used BK/β as a control in this experiment.

First, HEK293 cells co-expressing BKα-mCherry and BKβ1/-GFP were prepared by transfection of cDNA at a ratio of 1:1. The colocalization ratio of BKα-mCherry and BKβ1/-GFP was 86% (BKα alone: 14.2 ± 4.5% and BKα colocalized with BKβ1: 85.8 ± 4.5%, 119 particles from 12 cells). Single spots of BKβ1-GFP colocalized with BKα-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 0.75 (see “Materials and Methods”). This result provided evidence suggesting that each GFP spot contained four BK/β under these experimental conditions.

Second, HEK293 cells co-expressing BKα-mCherry and BKγ1/-GFP were prepared by the transfection with BKα + BKγ1 plasmid. The colocalization ratio of BKα-mCherry and BKγ1/-GFP was 81% (BKα alone: 19.1 ± 4.5% and BKα colocalized with γ1: 80.9 ± 4.5%, 194 particles from 7 cells). Similarly, the BKγ1/-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, BKγ1 can form tetramers with BKα at the stoichiometry of 1:1 in a similar manner as BK/β.

DISCUSSION

So far, two groups have suggested that a tetrameric BKα complex can interact with up to four BKγ1 using imaging and functional analyses. However, they did not directly count the number of BKγ1 within a single functional BKCa 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 BKγ1 within a single functional BKCa channel expressed in a human-derived cell line, HEK293 cells, using a TIRF microscope. Our data demonstrate that BKγ1 can stably interact with BKα at the stoichiometry of 1:1 within a single BKCa channel complex in HEK293 cells.

We previously reported that the mobility of BKα was markedly reduced by forming complexes with BKβ1/γ. Accordingly, we examined here whether BKγ1 also influences the mobility of BKα on the membrane surface. As shown in Fig. 2, BKα mobility was significantly reduced by forming complex with BKγ1 (Fig. 2). These results suggest that BKγ1 interacts with scaffold proteins through intracellular C-terminal, while the molecular entity of the scaffold proteins was not further examined in this study. As for BKβ1, it dynamically changes its intracellular localization in response to intracellular signaling of Ca2+ or NO in SMCs and regulates BKCa channel activity. The movement of BKγ1 between intracellular compartment and plasma membrane with respect to its influence on BKCa channel activity is an emergent topic and remains to be examined.

As shown in Fig. 3D, the distribution pattern of numbers of BKβ1 and BKγ1 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 BKα + BKβ1 and BKα + BKγ1 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 BKγ1 and BKα is supposed to be comparable with that between BKβ1 and BKα. It has been suggested that a BKCa channel can contain zero to four BKβ or BKγ per channel. The incorporation of one to four BKβ shows incremental effects on BKCa channel gating. In contrast, it has been suggested that BKγ1 affects BKCa channel gating in an all-or-none manner and that a single BKγ1 incorporation is enough to induce the full channel activation. However, the researchers used BKβ2/γ1 chimeric construct but not the native γ1 subunit. The present study, in which equal amount of cDNA of pore and auxiliary subunits are transfected in the expression system, showed that four BKβ1 or BKγ1 can be the most preferentially incorporated in each BKCa channel complex.

BKγ1 is thought to be a major BKCa channel subunit in smooth muscle tissues, such as vascular smooth muscles. Recently we demonstrated that BKγ1 expression is exceptionally high in mouse bronchial SMCs in comparison with other several types of SMCs and that the half activation voltage (V1/2) of BKCa channel current is close to that in BKα + BKγ1 in HEK293 reconstituted system. The physiological impact of the finding is that BKCa 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 BKβ1 to BKα + BKγ1 in HEK293 cells (BKα + BKβ1 + BKγ1) unexpectedly resulted in a slight shift of V1/2 to positive direction. Considering that both BKβ1 and BKγ1 are expressed in mouse bronchial SMCs, it is possible that both subunits simultaneously bind to the same BKα tetramer. In the previous and present studies, however, it was not quantitatively examined how BKβ1 and BKγ1 can be incorporated together in a single BKCa channel. Thus, the hot issue about the relationship between BKβ and BKγ, i.e., how these subunits influence the binding to BKα each, remains to be further determined.

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Conflict of Interest The authors declare no conflict of interest.

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