Biochemical and Biophysical Research Communications 380 (2009) 832–837

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Heteromeric assembly of inward rectifier channel subunit Kir2.1 with Kir3.1 and with Kir3.4

Keiko Ishihara [a,*], Tomomi Yamamoto [b], Yoshihiro Kubo [b,*]

[a] Department of Physiology, Faculty of Medicine, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan
[b] Division of Biophysics and Neurobiology, Department of Molecular Physiology, National Institute for Physiological Sciences, Myodaiji, Okazaki, Aichi 444-8585, Japan

ARTICLE INFO

Article history:
Received 28 January 2009
Available online 4 February 2009

Keywords:
Inward rectifier potassium channel
Heteromultimer
Co-localization
Immuno-precipitation

ABSTRACT

Heteromultimerization of different pore-forming subunits is known to contribute to the diversity of inward rectifier K^+ channels. We examined if the subunits belonging to different subfamilies Kir2 and Kir3 can co-assemble to form heteromultimers in heterologous expression systems. We observed co-immunoprecipitation of Kir2.1 and Kir3.1 as well as Kir2.1 and Kir3.4 in HEK293T cells. Furthermore, analyses of subcellular localization using confocal microscopy revealed that co-expression of Kir2.1 promoted the cell surface localization of Kir3.1 and Kir3.4 in HEK293T cells. In electrophysiological experiments, co-expression of Kir2.1 with Kir3.1 and/or Kir3.4 in *Xenopus* oocytes and HEK293T cells did not yield currents with distinguishable features. However, co-expression of a dominant-negative Kir2.1 with the wild-type Kir3.1/3.4 decreased the Kir3.1/3.4 current amplitude in *Xenopus* oocytes. The results indicate that Kir2.1 is capable of forming heteromultimeric channels with Kir3.1 and with Kir3.4.

Introduction

The pore-forming subunits of the inward rectifier K^+ (Kir) channel family include various members belonging to subfamilies Kir1–Kir7. The Kir channel pore is composed of four subunits, and heteromultimerization of different subunits are known. Most of the assemblies are within the same subfamily, such as Kir3.1/3.2, Kir3.1/3.4 [1,2], Kir2.1/2.2 [3], and Kir2.1/2.4 [4]. However, assemblies beyond the subfamily, e.g. Kir4.1/5.1 [5], Kir4.2/5.1 [6], and Kir4.1/2.1 [7], have also been reported. Here we focused on the heteromultimerization of the subunits from subfamilies Kir2 and Kir3. Kir2.1 is expressed in diverse tissues including those of the heart and brain and is one of the subunits forming the constitutively active strong inward rectifier K^+ channel [8]. Kir3.1 is also expressed in the heart and brain [9], and is known to assemble with Kir3.4 in the heart and with Kir3.2 in the brain to form functional channels [1,2]. In the heart, the channels formed with Kir2.1 carry the current I_{K1}, which plays a crucial role in stabilizing the resting potential of the ventricular and atrial myocytes. However, Kir2.1 has also been shown to be expressed in the sinoatrial node (SAN), the pacemaker of the heart [10,11], where a spontaneous depolarization of the resting potential is observed because I_{K1} is absent. Kir3.1 and Kir3.4, expressed mainly in SAN and atrial myocytes in the heart, form heterotetrameric channels, which are coupled to m2 muscarinic receptor via G-protein $\beta\gamma$ subunits and carry the ACh-activated K^+ current ($I_{K,ACh}$) regulating the heart rate [1,12]. Although the Kir3.4 knockout mice lacked $I_{K,ACh}$ in the heart [13], only about half of Kir3.4 subunits in atrial myocytes have been shown to form heteromers with Kir3.1, and the rest of Kir3.4 subunits may exist in homotetramers [14], of which physiological relevance is still unclear [15]. Thus, roles of individual Kir subunits remain to be fully understood. In this study, we examined the possibility that Kir2.1 forms heteromultimeric channels with Kir3.1 or Kir3.4, using heterologous expression systems. For this purpose, we analyzed associations and subcellular localizations of the subunits and electrophysiological properties of them in heterologous expression systems. Our results suggest that heteromultimeric assembly of Kir2.1/3.1 and Kir2.1/3.4 may occur in native tissues and may contribute to the functional diversity of Kir channels.

Materials and methods

Molecular biology. Original cDNA clones of mouse Kir2.1 [8], rat Kir3.1 [9], and rat Kir3.4 [16] were used. Rat $P2X_2$ cDNA [17] was provided by Dr. David Julius (UCSF, USA). cDNA for Cerulean (a brighter variant of CFP) was made based on the sequence information [18] and that for Venus (a brighter variant of YFP) [19] was provided by Dr. Atushi Miyawaki (RIKEN BSI, Japan).

FLAG-tag (DYKDDDDK) or myc-tag (QKLISEEDL) was attached to the C-terminus end of these channel proteins by polymerase chain reaction (PCR) using a high fidelity Taq polymerase KOD plus

* Corresponding authors. Fax: +81 952 34 2013 (K. Ishihara), +81 564 55 7825 (Y. Kubo).
E-mail addresses: keiko@med.saga-u.ac.jp (K. Ishihara), ykubo@nips.ac.jp (Y. Kubo).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.01.179

(TOYOBO, Japan) and a primer set encoding the N-terminus end region and the C-terminus end region with the tag sequence in frame.

Fluorescent protein (FP) (Cerulean or Venus) was attached to the C-terminus end of the channel proteins by overlapping extension PCR using KOD plus polymerase and a set of four primers. In the first step, a full length channel coding region ending with a sequence encoding four amino acids at the N-terminus end of FP were amplified. Also, a full length FP coding region starting with a sequence encoding four amino acids at the C-terminus end of channel protein was amplified. In the second step, the obtained two PCR products were used as a template and a primer each other, to extend the whole sequence of the coding region of channel-FP fusion protein, and then amplified by a set of primer encoding the N-terminus end of the channel protein and the C-terminus end of FP.

Triple point mutations to the pore region (GYG to AAA) were introduced also by overlapping extension PCR method using a set of four primers at the N-terminus end, C-terminus end, and two at the mutated region.

In all cases, DNA sequences of the modified region as well as the surrounding region were confirmed. The constructs were subcloned into a mammalian expression vector pCXN2 provided by Dr. Junichi Miyazaki (Osaka University, Japan) [20], which has a CMV enhancer and a β-actin promoter. For expression in *Xenopus* oocytes, cRNA was prepared from template plasmid DNA using an RNA transcription kit (Stratagene, USA). The concentration and integrity of cRNA was confirmed by agarose gel analysis.

Cell culture. HEK293T cells were cultured with DMEM (Nissui, Japan) supplemented with 10% Fetal Calf Serum, glutamine, penicillin, and streptomycin. For the co-immunoprecipitation experiment, cells of each group were grown in a dish of 6 cm diameter. For the confocal microscopy observations, cells were grown in glass-bottom dishes. Cells were transfected with plasmid DNAs using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

Confocal microcopy. After 1–2 days of plasmid transfection, the culture medium was replaced with standard saline solution supplemented with 5 mM glucose, and live cell images were obtained on an inverted confocal laser microscopy (LSM5 Pascal, Carl Zeiss, Germany). Optical sections were obtained using Plan-Apochromat 63×/1.4 oil immersion lens and a pinhole of 86 μm. Cerulean and Venus were excited by 458 nm and 514 nm argon laser, respectively, reflected by a main beam splitter HFT 458/514. Emission signals of Cerulean and Venus were split by a secondary beam splitter NFT 515 and were collected using 475–525 nm band-pass and 530 nm long-pass filters, respectively. The image analyses were done off-line using LSM Image Browser (Carl Zeiss). Signals of Cerulean and Venus in the figure are presented in a pseudo color.

Co-immunoprecipitation. After 2 days of plasmid transfection, cells from 6 cm dish were collected, suspended in 500 μl of PBS-based lysis buffer containing 1% triton, 0.5% deoxycholate, and Protease inhibitor mixture without EDTA (Roche, USA), lysed by sonification on ice, and the supernatant after centrifugation of 15,000g for 30 min was collected. Fifty microliters of it was separated and mixed with 25 μl of standard 3× SDS buffer with DTT. The rest was mixed with 15 μl of M2 anti-FLAG antibody agarose (Sigma, USA) and incubated at 4 °C for 90 min. The beads were then rinsed five times with PBS-based rinse buffer containing 1% triton and 0.5% deoxycholate by mild centrifugation of 3000*g* for 5 s.

The beads were then suspended with 100 μl of standard 1× SDS buffer with DTT. The samples were incubated at room temperature for 40 min, and then loaded on 4–20% gradient gel (Daiichi-kagaku, Japan). Proteins in the gel was transferred to PDVF membrane and detected by anti-FLAG antibody (Sigma, USA) or myc antibody (Sigma, USA), then by HRP-conjugated secondary antibodies, visualized by chemiluminescence reaction kit (Daiichi-kagaku), and digital images were recorded using LAS imager (Fuji, Japan).

Electrophysiology. Animal experiments in this study conformed to the guidelines of and approved by the Animal Experiment Committee of National Institute for Physiological Sciences, Japan.

Frogs were anesthetized using 0.15% Tricaine, and oocytes were obtained surgically. Isolated oocytes were dissociated by treating with 2 mg/ml collagenase (Sigma, USA) at room temperature for 5 h, and each oocyte was injected with 50 nl cRNA. Oocytes were incubated in frog ringer solution with penicillin and streptomycin at 17 °C for 2 days.

Macroscopic currents were recorded using the two-electrode voltage-clamp technique with a bath-clamp amplifier (OC-725C, Warner, USA). Stimulation, data acquisition and data analysis were done on a Pentium-based computer using Digidata 1322A and pCLAMP8 software (Axon Instruments, USA). Glass microelectrodes were filled with pipette solution containing 3 M potassium acetate and 10 mM KCl (pH 7.2). The resistance of the pipettes ranged from 0.1 to 0.2 MΩ. Recordings were obtained at room temperature (22–25 °C). The bath solution contained 90 mM KCl, 3 mM $MgCl_2$ and 5 mM Hepes (pH 7.35–7.4).

Data shown in a bar graph are the average and standard deviation. Statistical analyses were done by Students' unpaired *t*-test, and $p < 0.05$ were judged to be statistically significant.

Results and discussion

Co-immunoprecipitation experiments

We made FLAG or myc-tagged Kir2.1, Kir3.1, and Kir3.4 constructs and examined whether Kir2.1 associates with Kir3.1 and with Kir3.4 when co-expressed in HEK293T cells. Myc-tagged Kir2.1 (~53 kDa band in Fig. 1A, left four lanes) was co-immunoprecipitated with FLAG-tagged Kir3.1 or FLAG-tagged Kir3.4, at a comparable level with the positive control, FLAG-tagged Kir2.1 (Fig. 1B). This clear co-immunoprecipitation was not observed with FLAG-tagged $P2X_2$, even though strong signals were observed for myc-tagged Kir2.1 in the lysate (Fig. 1A) and for the immunoprecipitated FLAG-tagged $P2X_2$ (~70 kDa band in Fig. 1C). Furthermore, myc-tagged Kir3.1 (~60 kDa doublet bands and a 65–70 kDa band marked with asterisks in Fig. 1A, right four lanes) was co-immunoprecipitated with FLAG-tagged Kir2.1 at a comparable level with the positive control, FLAG-tagged Kir3.4 (Fig. 1B). It has been reported that the 65–70 kDa band is the glycosylated form of Kir3.1 [1], and association with Kir3.4 enables Kir3.1 lacking the ER export signal to traffic from the ER to the Golgi apparatus, where mature glycosylation occurs [21]. In clear contrast, myc-tagged Kir3.1, which was co-immunoprecipitated with FLAG-tagged Kir3.1, contained only the ~60 kDa doublet proteins (Fig. 1B), which reportedly consist of non-glycosylated and immature, core-glycosylated Kir3.1 proteins [22]. These findings suggested that Kir2.1 has the capability to form heteromultimeric channels with Kir3.1, thereby promoting the trafficking of Kir3.1 from the ER to the Golgi apparatus. When myc-tagged Kir3.1 was co-expressed with FLAG-tagged $P2X_2$, only a ~60 kDa single band was observed, indicating that the non-glycosylated form of Kir3.1 may have non-specifically associated with $P2X_2$ in the ER, but this Kir3.1/$P2X_2$ complex was not properly processed in the ER. Myc-tagged Kir3.4 was also co-immunoprecipitated with FLAG-tagged Kir2.1 (data not shown). These results suggested that Kir2.1 and Kir3.1 as well as Kir2.1 and Kir3.4 subunits associate to form heteromultimers in HEK293T cells.