Clustering and Enhanced Activity of an Inwardly Rectifying Potassium Channel, Kir4.1, by an Anchoring Protein, PSD-95/SAP90*  

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An inwardly rectifying potassium channel predominantly expressed in glial cells, Kir4.1/KAB-2, has a sequence of Ser-Asn-Val in its carboxy-terminal end, suggesting a possible interaction with an anchoring protein of the PSD-95 family. We examined the effects of PSD-95 on the distribution and function of Kir4.1 in a mammalian cell line. When Kir4.1 was expressed alone, the channel immunoreactivity was distributed homogeneously. In contrast, when co-expressed with PSD-95, prominent clustering of Kir4.1 in the cell membrane occurred. Kir4.1 was co-immunoprecipitated with PSD-95 in the co-expressed cells. Glutathione S-transferase-fusion protein of COOH terminus of Kir4.1 bound to PSD-95. These interactions disappeared when the Ser-Asn-Val motif was deleted. The magnitude of whole-cell Kir4.1 current was increased by 2-fold in cells co-expressing Kir4.1 and PSD-95 compared with cells expressing Kir4.1 alone. SAP97 (9) can interact with Kir4.1, resulting in clustering the Kir4.1 channel. In this study, the interaction between Kir4.1 and members of the PSD-95 family may affect the distribution and function of this channel. In this study, the interaction between Kir4.1 and PSD-95 members was studied. We show that PSD-95 and SAP97 (9) can interact with Kir4.1, resulting in clustering the channel proteins on the membrane and enhancing the Kir4.1 current in HEK293T cells. Because we also found that Kir4.1 and SAP97 clustered in retinal glial cells, the PSD-95 family of proteins may be a physiological regulator of the distribution and function of Kir4.1 in glial cells.

Distribution of ion channels in specialized areas of cell membrane is essential for various cell functions: for example, voltage-gated Na+ and K+ channels are concentrated at nodes of Ranvier for saltatory conduction in myelinated nerves (1). At the neuromuscular junction, the nicotinic acetylcholine receptor channels are concentrated at the postsynaptic membrane to initiate excitation-contraction coupling in skeletal muscle (2). Some molecular mechanisms responsible for the specific distribution of channel proteins have been characterized, e.g. agrin and rapamycin of the dystroglycan complex are involved in accumulation and clustering of nicotinic acetylcholine receptors (2). However, different molecular mechanisms may be responsible for clustering different channels. Recent studies using yeast two-hybrid method have revealed that clustering of voltage-gated K+ (3) and NMDA1 channels (4), was mediated by PSD-95 family proteins. PSD-95/SAP90 (5, 6) belongs to the membrane-associated anchoring proteins and is characterized with three PDZ domains in its amino terminus, an SH3 domain, and a carboxyl-terminal guanylate kinase homology domain. So far several members of the PSD-95 family have been cloned (7–9). The PDZ domains of PSD-95 and the Thr/Ser-X-Val motifs of the COOH termini of voltage-gated K+ and NMDA channels are supposed to interact for aggregation. Although co-localization of NMDA receptors and PSD-95 in cultured hippocampal neurons may indicate that these two proteins interact with each other in vivo (4), a physiological role for this interaction has not been fully elucidated.

Inwardly rectifying K+ channels comprise a family with more than ten members (10). These K+ channels play a pivotal role in determining resting membrane potential, in regulating action potential duration, and in transporting K+ ions. One inwardly rectifying K+ channel, Kir4.1/KAB-2, which we have cloned previously (11), has an amino acid motif of Ser-X-Val at its COOH terminus. The interaction of Kir4.1 and members of the PSD-95 family may affect the distribution and function of this channel. In this study, the interaction between Kir4.1 and PSD-95 members was studied. We show that PSD-95 and SAP97 (9) can interact with Kir4.1, resulting in clustering the channel proteins on the membrane and enhancing the Kir4.1 current in HEK293T cells. Because we also found that Kir4.1 and SAP97 clustered in retinal glial cells, the PSD-95 family of proteins may be a physiological regulator of the distribution and function of Kir4.1.

MATERIALS AND METHODS  

Transient Expression of cDNAs in HEK293T Cells—Rat Kir4.1 (11) was transfected with LipofectAMINE (Life Technologies, Inc.) into HEK 293T cells as described previously (12). Rat PSD-95 cDNA was tagged2 with 110 amino acid residues at the COOH terminus of bovine carboxylase carrier protein (BCCP) cDNA (13) and introduced into pCMV5 (kindly provided by Dr. D. E. Russell). For electrophysiological experiments, green fluorescent protein plasmid (14) was co-transfected with Kir4.1 cDNA.  

Electrophysiological Recordings—Whole-cell and single-channel currents of HEK cells were measured as described (12).

Solutions and Chemicals—In whole-cell experiments, the bathing solution contained (in mM): 135 NaCl, 5.4 KCl, 1.8 CaCl2, 1.2 MgCl2, 10 HEPES, 10 glucose, pH 7.4. In current-clamp experiments, the patch pipette solution contained: 130 KCl, 10 HEPES, 10 glucose, pH 7.3.  

The abbreviations used are: NMDA, N-methyl-D-aspartate; BCCP, biotin carboxylase carrier protein; DMEM, Dulbecco’s modified Eagle’s medium; FITC, fluorescein isothiocyanate; GST, glutathione S-transferase; HEK, human embryonic kidney; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; F, farad(s); S, Siemens.  

1 This paper is available on line at http://www-jbc.stanford.edu/jbc/
solution contained (in mM): 40 KCl, 100 NaCl, 1.8 CaCl₂, 0.53 MgCl₂, 5.5 glucose, and 5.5 HEPES-KOH, pH 7.4. The pipette solution contained (in mM): 120 KCl, 5 K₂ATP, 1 MgCl₂, 5 EGTA, and 5 HEPES-KOH, pH 7.3. In single-channel recordings, the pipette solution contained (in mM): 140 KCl, 1 CaCl₂, 1 MgCl₂, and 5 HEPES-KOH, pH 7.4. The bath was perfused with the solution composed of (in mM): 140 KCl, 5 EGTA, and 5 HEPES-KOH, pH 7.3, with 2 MgCl₂.

Antibody—Anti-Kₐ₋₂A₁ antibody and anti-Kₐ₋₂C₂ antibody were raised in rabbits against synthetic peptides corresponding to amino acids 13–26 in the amino-terminal region and amino acids 266–379 in the COOH terminus of rat Kir4.1, respectively. Both antibodies were purified with antigenic peptide-coupled Sulfolink resin (Pierce) (15).

Immunocytochemistry—For immunocytochemical experiments, the anti-Kₐ₋₂C₂ antibody was used. Identification of Müller cells, monoclonal anti-vimentin antibody (Zymed Laboratory, San Francisco, CA) was used. Efficiencies of PSD-95 and PSD-95-BCCP in clustering Kir4.1 immunoreactivity were essentially the same. Anti-SAP97 antibody was raised in rabbit. Cells were examined with a confocal microscope (MRC-1024, Bio-Rad, Hertfordshire, United Kingdom).

Biochemical Analysis—Fusion proteins with glutathione S-transferase (GST) of Kir 4.1 amino acids 165–376 (GST-Kir4.1C) or amino acids 165–379 (GST-Kir4.1C) were expressed in Escherichia coli and purified on glutathione-Sepharose. HEK cells transfected with PSD-95 were homogenized and solubilized in a lysis buffer (40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.2 mg/ml benzamidine, 30 kilounits/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100) and then centrifuged at 100,000 × g for 30 min at 4 °C. Solubilized homogenates were incubated for 2 h with 2 μg of purified fusion protein bound to 20 μl of glutathione-Sepharose. Samples were washed four times with phosphate-buffered saline (PBS) containing 0.4 M NaCl, resolved by SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE), and then analyzed by Western blot using anti-PSD-95 antibody (Transduction Laboratories, Lexington, KY). For immunoprecipitation, Kir4.1 (amino acids 1–379) or Kir4.1 (amino acids 1–376), which lacks the Ser-Asn-Val motif at the COOH-terminal end, were expressed with PSD-95 in HEK cells. Solubilized proteins of the cells (1 × 10⁶ cells) were incubated with anti-Kₐ₋₂A₁ antibody (4 μg/ml) overnight at 4 °C. 50 μl of protein A-Sepharose was added to the solution. Protein A-Sepharose-bound material was resolved by SDS-PAGE and probed with anti-PSD-95 antibody.

Preparation of Isolated Cells—Müller cells were isolated from retinas of Wistar rats (Nippon Doubutsu, Kyoto, Japan) (16).

RESULTS
To investigate the interaction between Kir4.1 and PSD-95, we transiently expressed Kir4.1 and PSD-95-BCCP in HEK cells and examined the distribution immunocytochemically. The anti-Kir4.1 antibody (anti-KAB-2C2) (15) was used. The distribution of Kir4.1 immunoreactivity was homogenous in most cells transfected with Kir4.1 alone (green in Fig. 1A). Prominent clustering of Kir4.1 occurred when PSD-95 was co-expressed (arrows in Fig. 1B). In the cells showing clustered distribution of Kir4.1, the PSD-95 protein was always co-expressed (red in Fig. 1C). Double staining showed that Kir4.1 and PSD-95 co-localized in close vicinity (yellow in Fig. 1D).

Because Kir4.1 and PSD-95 were transiently expressed, some cells expressed only Kir4.1. In Fig. 1B, diffuse staining of Kir4.1 was detected in two cells (arrowheads), where the expression of PSD-95 was not detected (arrowheads in Fig. 1C). Furthermore, when PSD-95 was expressed alone, it distributed diffusely (Fig. 1F). These results suggest that there is a reciprocal relationship between Kir4.1 and PSD-95 on the cell membrane.

SAP97, another member of the PSD-95 family, when co-transfected with Kir4.1 clustered the channel proteins (Fig. 1E). In these cells, SAP97 itself was also clustered (Fig. 1F), although it distributed diffusely when transfected alone (Fig. 1H). Because both of the antibodies against Kir4.1 and SAP97 were raised in rabbit, double staining of Kir4.1 and SAP97 could not be performed. These results, however, strongly suggest that SAP97 also co-localized with Kir4.1 and clustered the channel protein on the cell membrane.

Fig. 1. Clustering of Kir4.1 by PSD-95/SAP90 in HEK293T cells.
A–D, Kir4.1 was transiently expressed without or with PSD-95-BCCP. The cells were stained with the anti-Kₐ₋₂C₂ antibody and then with FITC-conjugated anti-rabbit IgG and Texas Red-labeled streptavidin. Kir4.1 immunoreactivity (green) and staining of PSD-95 (red) were shown. A, without PSD-95, diffuse and homogenous immunoreactivity of Kir4.1 (green) was detected. B–D, cells expressing both Kir4.1 and PSD-95 showed clustering of Kir4.1 (arrows), but cells expressing Kir4.1 alone showed diffuse and smooth staining (arrowheads). D, double exposure of both images. E and F, cells transfected with Kir4.1 and SAP97 were stained with anti-Kₐ₋₂C₂ antibody and FITC-labeled anti-rabbit IgG (E) or anti-SAP97 antibody and Texas Red-labeled anti-rabbit IgG (F). Both Kir4.1 and SAP97 clustered on the membranes. G and H, cells transfected with PSD-95-BCCP alone (G) and with SAP97 alone (H) were stained with Texas Red-labeled streptavidin and anti-SAP97 antibody plus Texas Red-labeled anti-rabbit IgG, respectively. Both PSD-95 and SAP97 distributed diffusely. Scale bars: 10 μm.

To examine the molecular mechanism responsible for interaction between Kir4.1 and PSD-95, a mutant COOH terminus of Kir4.1 (amino acids 165–376), which lacks Ser-Asn-Val motif, and the normal COOH-terminal region of Kir4.1 (amino acids 165–379) were expressed in E. coli with GST as fusion proteins (Fig. 2A, GST-Kir4.1C and GST-Kir4.1C, respectively). The fusion protein was bound to glutathione-Sepharose and incubated with the lysate of HEK cells, which had been transfected with PSD-95. The bound material was resolved by SDS-PAGE and subjected to Western blot analysis using anti-PSD-95 antibody. PSD-95 in the lysate did not associate with GST-Kir4.1C (Fig. 2B, GST-Kir4.1C/PSD-95) but did so with GST-Kir4.1C (Fig. 2B, GST-Kir4.1C/PSD-95). Such binding was not detected when the lysate of mock-transfected cells was used (Fig. 2B, GST-Kir4.1C control). To determine whether the interaction of Kir4.1 with PSD-95 occurs in cells, co-immunoprecipitation experiments were performed. Kir4.1Δ (amino acids 1–376) or the control Kir4.1 was expressed with PSD-95 in HEK cells. Expression of the channel proteins in these cells could be confirmed by anti-Kₐ₋₂A₁ antibody (Fig. 2A, Kir4.1Δ and Kir4.1). Immunoprecipitants from these cells...
The inwardly rectifying K⁺ channel by PSD-95/SAP90

The interaction of PSD-95 family and Kir4.1 might also occur in vitro. To examine this possibility, we studied cellular distribution of Kir4.1 in retinal glial cells (Müller cells) (11, 17). Isolated Müller cells were stained with the anti-Kir4.1 antibody. The immunoreactivity of Kir4.1 (Fig. 4B) clustered on the membrane of cells, which were stained with vimentin, a marker of Müller cells (Fig. 4B). Reverse transcriptase-polymerase chain reaction using mRNA of an isolated single Müller cell and specific primers for the second PDZ domain of PSD-95 resulted from the increase in the number of functional Kir4.1 channels in the membrane.

The interaction of PSD-95 family and Kir4.1 might also occur in vivo. To examine this possibility, we studied cellular distribution of Kir4.1 in retinal glial cells (Müller cells) (11, 17). Isolated Müller cells were stained with the anti-Kir4.1 antibody. The immunoreactivity of Kir4.1 (Fig. 4A) clustered on the membrane of cells, which were stained with vimentin, a marker of Müller cells (Fig. 4B). Reverse transcriptase-polymerase chain reaction using mRNA of an isolated single Müller cell and specific primers for the second PDZ domain of PSD-95 family showed that at least SAP97 was expressed in the cell.

Fig. 2. Interaction of Kir4.1 and PSD-95/SAP90. A, fusion proteins of GST fused to amino acids 165–376 (GST-Kir4.1C) or amino acids 165–379 (GST-Kir4.1CΔ) of Kir4.1 were expressed in E. coli. Expressed fusion proteins were purified on glutathione-Sepharose and were analyzed by SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue (left panel). Kir4.1A (amino acids 1–376) and Kir4.1 (amino acids 1–379) expressed in HEK293T cells were solubilized and then analyzed by Western blot using anti-KAB-2A1 antibody (right panel). Both Kir4.1A and Kir4.1 were recognized by the anti-KAB-2A1 antibody. B, PSD-95 did not interact with GST-Kir4.1C (GST-Kir4.1C PSD-95) but was bound to GST-Kir4.1C (GST-Kir4.1C PSD-95). GST-Kir4.1C did not interact with the lysate of mock-transfected HEK293T cells (GST-Kir4.1C control). For immunoprecipitation experiments, solubilized proteins from cells transfected Kir4.1A plus PSD-95 or Kir4.1 plus PSD-95 was immunoprecipitated with anti-KAB-2A1 antibody and then analyzed by SDS-PAGE and Western blot using anti-PSD-95 antibody. PSD-95 was not co-immunoprecipitated with Kir4.1A (Kir4.1A+PSD-95 IP) but was precipitated with Kir4.1 (Kir4.1+PSD-95 IP).

using anti-KAB-2A1 were separated by SDS-PAGE and immunoblotted with anti-PSD-95 antibody. The immunoprecipitant from the cells expressing Kir4.1 plus PSD-95 (Fig. 2B, Kir4.1+PSD-95 IP), but not from those expressing Kir4.1 plus PSD-95 (Fig. 2B, Kir4.1A+PSD-95 IP), contained the PSD-95 band. These results indicate that PSD-95 may form a macromolecular complex with Kir4.1 and that the Ser-Asn-Val motif of Kir4.1 is indispensable for the interaction.

PSD-95 may also affect the function of Kir4.1. Using the patch clamp technique, we compared Kir4.1 channel activity in HEK cells co-expressed with or without PSD-95 (Fig. 3). In the whole-cell clamp mode, the membrane currents elicited by command steps were compared (Fig. 3A). Both cells expressed K⁺ currents which were blocked by 1 mM Ba²⁺ (Fig. 3B). In the control cells or those transfected with only PSD-95, the Ba²⁺-sensitive K⁺ current was more than two times larger than that observed in those transfected with Kir4.1 alone. At −120 mV, the Ba²⁺-sensitive K⁺ current was 232.6 ± 56.3 pA/pF in those transfected with both Kir4.1 and PSD-95 (n = 6), and 194.3 ± 50.2 pA/pF in the cells expressing Kir4.1 alone (n = 11, p < 0.001, Fig. 3C). When Kir4.1 was co-transfected with SAP97, the K⁺ current was more than three times larger than that observed with Kir4.1 alone (428.9 ± 64.7 pA/pF, n = 7, p < 0.001, Fig. 3C).

The single channel properties of Kir4.1 in the cells transfected with both Kir4.1 and PSD-95 and in those with Kir4.1 alone did not differ from each other (Fig. 3, D and E). The unitary channel conductances of Kir4.1 alone, Kir4.1 plus PSD-95 and Kir4.1 plus SAP97 were 22.1 ± 1.4 pS (n = 3), 20.6 ± 1.0 pS (n = 3), 21.7 ± 0.8 pS (n = 3), respectively. Open probabilities of the Kir4.1 channel with and without PSD-95 family proteins were constant −0.9 between −40 and −100 mV (data not shown). These results suggest that the larger current in the cells co-expressing Kir4.1 and PSD-95 or SAP97 has resulted from an increase in the number of functional Kir4.1 channels in the membrane.

Fig. 3. Increase in whole-cell Kir4.1 current amplitude in cells co-transfected with PSD-95/SAP90. A, whole-cell currents of HEK cells transfected with Kir4.1 in the absence or presence of PSD-95. Currents were recorded with voltage steps from −120 mV to +40 mV in 10-mV steps. Holding potential was −30 mV. Measured whole-cell current was normalized by dividing by cell capacitance. B, examples of current-voltage relationships (I-V relationship) of the cells expressing Kir4.1 with or without PSD-95. The steady-state current amplitudes measured 500 ms after the start of voltage pulses are plotted. Circles show the I-V relationships of the cell transfected with Kir4.1 alone in the presence (open circle) or absence (filled circle) of 1 mM Ba²⁺. Squares show I-V relationships of the cell transfected with Kir4.1 plus PSD-95 in the presence (open square) or absence (filled square) of Ba²⁺. C, Ba²⁺-sensitive whole-cell current amplitudes evoked by −120 mV voltage command. Data are shown as means ± S.D. Both current amplitudes from cells transfected with Kir4.1 plus PSD-95 and Kir4.1 plus SAP97 were significantly different from cells transfected with Kir4.1 alone (*, p < 0.001) using Anova test. D, single-channel recordings from cells attached membrane patches of cells expressing Kir4.1 or Kir4.1 plus PSD-95. The membrane potential was −60 mV. The zero current level is indicated as a dotted line. E, examples of single-channel current-voltage relationships of cells expressing Kir4.1 alone (circles), Kir4.1 plus PSD-95 (squares), and Kir4.1 plus SAP97 (triangles). Single channel properties of Kir4.1 were not altered by the expression of PSD-95 or SAP97.
Thus, we stained Müller cells with anti-SAP97 antibody (Figs. 4, D–F). SAP97 was expressed and clustered in Müller cells in a similar manner to Kir4.1. It is, therefore, strongly suggested that SAP97 might be responsible for aggregation of Kir4.1 in Müller cells.

**DISCUSSION**

The major findings of this study are 1) Kir4.1 was shown to interact with PSD-95 through the Ser-Aan-Val motif; 2) the PSD-95 family proteins caused not only clustering of Kir4.1 on the cell membrane, but also increased the number of functional channels; 3) this interaction may occur in vivo and might be important for the physiological function of Kir4.1 channels.

The co-localization of Kir4.1 and PSD-95 in the cell membrane may result from direct interaction between these proteins, because PSD-95 bound to the GST-fusion protein of Kir4.1 C terminus and, moreover, was co-immunoprecipitated with Kir4.1. Because both interactions disappeared when the Ser-Aan-Val motif was deleted from Kir4.1, the motif is indispensable for Kir4.1-binding to PSD-95. The interaction is not, however, specific to PSD-95 itself, because SAP97 could also cause clustering of Kir4.1.

PDZ domains in various proteins can interact with each other, e.g., the PDZ domain of neuronal nitric-oxide synthase interacts with the second PDZ of PSD-95, and nitric-oxide synthase and skeletal muscle syntrophin also interact through their PDZ domains (18). Thus, PSD-95 proteins might self-aggregate through their PDZ domains. However, because PSD-95 and SAP97 diffusely distributed when transfected alone (Figs. 1, G and H), the formation of a protein complex with Kir4.1 might be essential for their aggregation.

Co-expression of Kir4.1 with PSD-95 or SAP97 prominently enhanced the whole-cell Kir4.1 currents. Because single-channel properties of Kir4.1 were not changed by the expression of PSD-95 or SAP97, an increase in the functional number of Kir4.1 channels should be mainly responsible for the increase. An increase in functional channel number can be achieved either by increasing the number of Kir4.1 proteins in the membrane and/or by facilitating formation of functional channels. hdlg, a member of the PSD-95 family, was reported to bind band 4.1 protein, which co-localizes with actin (8). Thus, Kir4.1 forming a macromolecule complex with PSD-95 might be linked to the cytoskeletal matrix through associated proteins and could be stabilized in the membrane. Another possibility is that the PSD-95 family might facilitate formation of functional Kir4.1 tetramers, because the tetrameric structure is essential for channel function (19). Through such a mechanism, even if the total number of monomer subunit proteins expressed was not increased, the number of functional tetramers could be increased. At present, we cannot discriminate these possibilities. To elucidate the molecular mechanism of enhancement of Kir4.1 channel activity by PSD-95 family proteins, further studies are needed.

We found clustered distribution of Kir4.1 on the membrane of retinal Müller glial cells. Thus, clustering of Kir4.1 occurs in vivo. Immunostaining also showed that SAP97 was clustered in Müller cells. SAP97 might, thus, be responsible for clustering of Kir4.1 in Müller cells. Because Kir 4.1 is the dominant Kir channel in glial cells, Kir4.1 is considered to play a major role in spatial buffering of K+ ions, which regulates extracellular K+ ions released by synaptic excitations in brain and retina (20). The clustering and enhanced function of Kir4.1 mediated by PSD-95 family proteins may be essential for rapid K+ exchange between glial cells and neurons.

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