Anchoring proteins confer G protein sensitivity to an inward-rectifier K⁺ channel through the GK domain

Hiroshi Hibino, Atsushi Inanobe, Masayuki Tanemoto, Akikazu Fujita, Katsumi Doi¹, Takeshi Kubo¹, Yutaka Hata², Yoshimi Takai^{2,3} and Yoshihisa Kurachi⁴

Departments of Pharmacology II, ¹Otolaryngology and ³Molecular Biology and Biochemistry, Graduate School of Medicine, Osaka University, 2-2, Yamada-oka, Suita, Osaka 565-0871 and ²Takai Biotimer Project, ERATO, Japan Science and Technology Corporation, c/o JCR Pharmaceuticals Co. Ltd, 2-2-10 Murotani, Nishi-ku Kobe 651-2241, Japan

⁴Corresponding author
e-mail: ykurachi@pharma2.med.osaka-u.ac.jp

Anchoring proteins cluster receptors and ion channels at postsynaptic membranes in the brain. They also act as scaffolds for intracellular signaling molecules including synGAP and NO synthase. Here we report a new function for intracellular anchoring proteins: the regulation of synaptic ion channel function. A neuronal G protein-gated inwardly rectifying K⁺ channel, Kir3.2c, can not be activated either by M₂-muscarinic receptor stimulation or by G_{βγ} overexpression. When coexpressed with SAP97, a member of the PSD/SAP anchoring protein family, the channel became sensitive to G protein stimulation. Although the C-terminus of Kir3.2c bound to the second PDZ domain of SAP97, functional analyses revealed that the guanylate kinase (GK) domain of SAP97 is crucial for sensitization of the Kir3.2c channel to G protein stimulation. Furthermore, SAPAP1/GKAP, which binds specifically to the GK domain of membrane-associated guanylate kinases, prevented the SAP97-induced sensitization. The function of a synaptic ion channel can therefore be controlled by a network of various intracellular proteins.

Keywords: anchoring proteins/channel function/G protein sensitivity/inwardly rectifying K⁺ channel/Kir3.2c subunit

Introduction

The specific localization of ion channels, receptors and signaling molecules in the postsynaptic region is essential for effective synaptic transmission. The targeting of these proteins to the postsynaptic membrane is achieved by different anchoring and scaffold proteins, such as PSD-95, SAP97 and syntrophins (for review, see Froehner, 1993; Hall and Sanes, 1993; Saras and Heldin, 1996; Sheng, 1996; Ziff, 1997; Craven and Brecht, 1998; Gee *et al.*, 1998). However, the functional roles of these anchoring proteins on synaptic signal-transduction have not been fully elucidated.

G protein-gated K⁺ (K_G) channels are directly activated

by G proteins and coupled to various inhibitory receptors, including M₂-muscarinic, A₁-purinergic, D₂-dopaminergic, 5-HT_{1A}, μ- and δ-opioid, GABA_B and somatostatin receptors (Yamada *et al.*, 1998). Thus, they play a key role in generation of slow inhibitory postsynaptic potentials in the brain (Lüscher *et al.*, 1997). We recently found that K_G channels in dopaminergic neurons of substantia nigra are localized specifically at the postsynaptic membrane of the dendrites (Inanobe *et al.*, 1999). These K_G channels are either heteromultimers of Kir3.2a and Kir3.2c or homomultimers of Kir3.2c alone. However, when the K_G channels composed of Kir3.2c alone were expressed in *Xenopus* oocytes, they did not respond to G protein stimulation at all, although the channel proteins seemed to be generated and transported to the cell membrane as judged from the localization of green fluorescence protein (GFP) tagged to Kir3.2c (Inanobe *et al.*, 1999). Therefore, the homomeric Kir3.2c channel would be produced *in vivo* but not be functional. It is, however, also possible that unidentified mechanisms might exist that confer functionality to the deaf K_G channel.

In this study, because Kir3.2c could bind to PSD-95 and SAP97 proteins (Inanobe *et al.*, 1999), we hypothesized that the anchoring proteins might affect the function of Kir3.2c channels, and found that they confer the G protein sensitivity to homomeric Kir3.2c channels. Furthermore, the guanylate kinase (GK) domain of the anchoring proteins was indispensable for the SAP97-induced sensitization of Kir3.2c. Therefore, the anchoring proteins may regulate not only the distribution but also the function of synaptic ion channels in the brain.

Results

The effects of SAP97 on the K_G channels composed of Kir3.2c

To investigate the function of K_G channels composed of Kir3.2c alone, we injected the cRNA of Kir3.2c into *Xenopus* oocytes with either that of M₂-muscarinic receptor (M₂R) or those of G protein β1 and γ2 subunits (G_{βγ}). These oocytes exhibited neither background nor acetylcholine (ACh)-induced Kir currents, although the GFP fluorescence suggested that Kir3.2c proteins were localized at the plasma membrane (Figure 1A). The postembedding immunogold electron microscopy further showed that the gold particles of Kir3.2c immunoreactivity were associated with the invaginated plasma membrane of oocytes (Figure 1A, right panel). When we further coinjected the cRNA of either SAP97 or PSD-95 into the M₂R oocytes, the oocytes expressed a significant Ba²⁺-sensitive background Kir current, which was markedly enhanced by ACh (10 μM). The amplitude of the Ba²⁺-sensitive Kir current in the presence of 10 μM ACh was 5.81 ± 0.65 μA (n = 4) at -120 mV for the SAP97-injected oocytes

(Figure 1) and $5.70 \pm 0.55 \mu\text{A}$ ($n = 4$) for those injected with PSD-95. G $\beta\gamma$ could also fully activate the Kir3.2c channel in the presence of SAP97 ($6.45 \pm 1.02 \mu\text{A}$, $n = 4$; Figure 1). The intensity and distribution of Kir3.2c-GFP fluorescence as well as the density of Kir3.2c immunoreactivity detected with gold particles were not significantly affected by coexpression of either SAP97 (Figure 1A) or PSD-95 (data not shown). Therefore the coexpression of anchoring proteins was required for the full functional expression of Kir3.2c ion currents, which were activated by muscarinic receptor stimulation or more directly by overexpression of G $\beta\gamma$. During this series of experiments for >6 months, we obtained this effect of SAP97 and PSD-95 on the Kir3.2c channel in oocytes from most frogs, but in some frogs we could not see the effect. Therefore, in the following experiments, we always performed the control experiments using wild-type SAP97 or PSD-95 to confirm the oocytes being used to exhibit

the SAP97 or PSD-95-induced sensitization of Kir3.2c to G protein stimulation.

Kir3.2c possesses the PDZ domain binding motif, ESKV, at its C-terminus. The interaction between the C-terminus of Kir3.2c and PDZ-containing anchoring proteins might therefore be involved in the sensitization of Kir3.2c to G protein stimulation. Kir3.2a and Kir3.2c are splicing variants derived from a single gene where Kir3.2c is 11 amino acids longer than Kir3.2a at its C-terminus (Figure 1). This region is the only divergent part of the two isoforms and contains the ESKV sequence. Kir3.2a did not bind to PSD-95 or SAP97 (Inanobe *et al.*, 1999; see also Figure 2B). The oocytes injected with Kir3.2a cRNA expressed background and ACh (10 μM)-induced Kir currents even without coexpression of either SAP97 or PSD-95. The Ba²⁺-sensitive component of the Kir3.2a channel in the presence of ACh was $6.23 \pm 0.75 \mu\text{A}$ ($n = 4$) (Figure 1). G $\beta\gamma$ also fully activated

Fig. 1. Functional expression of Kir3.2 isoforms in *Xenopus* oocytes. (A) Left, whole-cell currents recorded from *Xenopus* oocytes expressing Kir3.2c, Kir3.2c plus SAP97, and Kir3.2a with either M₂-muscarinic receptor (M₂R) or G protein β 1 and γ 2 subunits (G $\beta\gamma$) indicated to the left of each family of traces. The bath solution contained 90 mM K⁺. Traces show current records obtained in the absence (Basal), in the presence of 10 μM acetylcholine (ACh) and after the addition of 1 mM Ba²⁺ (Ba²⁺) on M₂R-oocytes, and on G $\beta\gamma$ -oocytes (G $\beta\gamma$), as indicated above each column of current traces. Middle, the distribution of GFP fluorescence in the oocytes. The sections were obtained from the oocytes injected with the various combinations of cRNAs of GFP-tagged Kir3.2c or Kir3.2a and SAP97 indicated in each panel. Scale bar, 30 μm . Right, immunogold labeling of Kir3.2c in the oocytes coexpressed without or with SAP97. Dense particles of Kir3.2c proteins were found at the plasma membrane with microvilli, regardless of the expression of SAP97. Low density of the immunogold labeling was observed in the cytoplasm. Scale bars, 500 nm. CG, cortical granule. (B) Left, the amplitude of Kir currents recorded in oocytes injected with cRNAs of Kir3.2c, Kir3.2c plus SAP97, and Kir3.2a. Each column represents the average current amplitude ($n = 4$) at the end of the voltage step to -120 mV. The amplitude of each Kir current was obtained as the Ba²⁺ (1 mM)-sensitive component in the presence of 10 μM ACh for the oocytes that expressed M₂R (open column), or in the absence of ACh for the oocytes that coexpressed G $\beta\gamma$ (closed column). The error bars represent SE. Right, schematic representation of the structural difference between two Kir3.2 isoforms. The last four amino acids of Kir3.2c are responsible for the binding activity to SAP97.

the Kir3.2a channel ($6.45 \pm 0.6 \mu\text{A}$, $n = 4$; Figure 1). The coexpression of SAP97 or PSD-95 did not affect and certainly did not further increase the ACh- or $G_{\beta\gamma}$ -induced Kir3.2a currents ($n = 4$ for each; data not shown). We constructed a deletion mutant of Kir3.2c that lacked the final four ESKV amino acids of the C-terminus. When Kir3.2c- ΔC4 was expressed in oocytes, the properties of the ion currents generated were identical to those of Kir3.2a. The amplitude of the Ba^{2+} -sensitive Kir current of Kir3.2c- ΔC4 in the presence of ACh ($10 \mu\text{M}$) was $6.47 \pm 0.29 \mu\text{A}$ ($n = 4$). Thus, the four amino acids, ESKV, at the C-terminus are responsible for the insensitivity of the Kir3.2c channel to G protein stimulation in the absence of the anchoring proteins.

Identification of critical domains in SAP97-induced sensitization of Kir3.2c to G protein.

To examine which region(s) of the anchoring proteins is crucial for the sensitization of Kir3.2c to G protein stimulation, we concentrated upon SAP97. We constructed different truncated mutants of SAP97 and coexpressed them with Kir3.2c in the presence of either M_2R or $G_{\beta\gamma}$ in *Xenopus* oocytes (Figure 2A). Although with the wild-type SAP97, Kir3.2c was consistently activated by M_2R stimulation or $G_{\beta\gamma}$ overexpression, none of the truncated mutants of SAP97 that lacked potential Kir3.2c-interacting

PDZ domain(s), i.e. $\Delta\text{PDZ-1}$, $\Delta\text{PDZ-12}$, $\Delta\text{PDZ-123}$, SH3 and GK, could confer G protein sensitivity to the Kir3.2c channel (Figure 2A). Unexpectedly, the sensitization of Kir3.2c was also not achieved by coexpression of either PDZ-123 or ΔGK , which contained all the PDZ domains but not the GK domain. Therefore, to examine the actual interactions between Kir3.2c and SAP97, we performed a 'pull-down' assay in HEK293T cells (Figure 2B).

SAP97 expressed in HEK293T cells bound to the C-terminal region of the Kir3.2c glutathione *S*-transferase (GST) fusion protein (GST-Kir3.2c-C) (Figure 2Ba). This interaction was abolished when the four amino acids (ESKV) at the C-terminus of Kir3.2c were truncated (GST-Kir3.2c- ΔC4 -C). Neither the GST fusion protein of the C-terminus of Kir3.2a (GST-Kir3.2a-C) nor that of its N-terminus (GST-Kir3.2a/c-N) bound to SAP97. Next we examined the interaction between Kir3.2c and different parts of SAP97 (Figure 2Bb). The GST fusion protein of full-length SAP97 (GST-full) clearly bound to Kir3.2c. Consistent with the electrophysiological results (see Figure 2A), this interaction was abolished when the PDZ domains were deleted from SAP97 (GST- $\Delta\text{PDZ-12}$ and GST- $\Delta\text{PDZ-123}$). Of the three PDZ domains of SAP97, Kir3.2c bound to the second PDZ domain (GST-PDZ-2) but to neither the first nor the third domain (GST-PDZ-1 and GST-PDZ-3). In addition, Kir3.2c did not bind to either the SH3 domain (GST-SH3) or the GK domain (GST-GK) of SAP97. We also found no binding between SAP97 and either M_2R , G_{α} or $G_{\beta\gamma}$ under our experimental

Fig. 2. (A) The functional effects of deletion mutants of SAP97 on Kir3.2c in *Xenopus* oocytes. The deletion mutants of SAP97 used in this study are indicated in the left panel in (Aa). Kir currents recorded in the oocytes injected with cRNAs of Kir3.2c with the respective SAP97 mutants are indicated in the right panel in (Aa). Each column represents the current amplitude ($n = 4$) at the end of the voltage step to -120 mV. The amplitude of each Kir current was obtained as the Ba^{2+} (1 mM)-sensitive component in the presence of $10 \mu\text{M}$ ACh in the oocytes expressing M_2R (open column) or in the absence of ACh in the oocytes that coexpressed $G_{\beta\gamma}$ (closed column). The horizontal bars indicate the SE. (Ab) Representative traces of Kir current in M_2R oocytes injected with cRNAs of Kir3.2c plus SAP97, $\Delta\text{PDZ-12}$ or ΔGK . (B) Biochemical interaction between Kir3.2c and SAP97 shown by the 'pull-down' assay. (Ba) Fusion proteins of GST and cytoplasmic C-terminus of Kir3.2c (GST-Kir3.2c-C), Kir3.2c- ΔC4 (GST-Kir3.2c- ΔC4), Kir3.2a (GST-Kir3.2a-C), cytoplasmic N-terminus of Kir3.2c/Kir3.2a (GST-Kir3.2a/c-N) and GST alone on glutathione-Sepharose were incubated with the cell lysate expressed SAP97. After washing the resin, SAP97 retained in the resin was immunoblotted with anti-dlg antibody. (Bb) Interaction of different GST-fusion proteins of SAP97 deletion mutants with Kir3.2c in HEK293T cells. Immunoblotting was performed using anti-G2A-5 antibody. (Bc) Dominant-negative effects of mutants of SAP97 on K_G channels composed of Kir3.2c plus SAP97 in *Xenopus* oocytes. The deletion mutant of SAP97 is indicated in the left panel in (Ca). The right panel in (Ca) shows Kir currents recorded from *Xenopus* oocytes where the upper two columns show the control experiments, i.e. Kir3.2c alone and Kir3.2c plus SAP97. The lower three columns indicate the measurement of Kir currents recorded from oocytes expressing Kir3.2c plus SAP97 and overexpressed with each of the deletion mutants of SAP97 ($\Delta\text{PDZ-123}$, SH3 and GK). All oocytes were injected with cRNA of M_2R . In this experiment, cRNA of SAP97-mutants was injected at 4-fold higher concentration than that of wild-type SAP97 (lower three columns). Each column represents the average current amplitude ($n = 3$) at the end of the voltage step to -120 mV. The amplitude of each Kir current was obtained as the Ba^{2+} (1 mM)-sensitive component in the presence of $10 \mu\text{M}$ ACh. The vertical bars indicate the SE. (Cb) Representative traces of Kir current in oocytes injected with cRNAs of Kir3.2c plus SAP97 and Kir3.2c, SAP97 plus SH3 or ΔGK .

conditions (data not shown). These results suggest that Kir3.2c specifically binds to the second PDZ domain of SAP97 through the four amino acids at its C-terminus.

Because the truncated forms of SAP97, PDZ-123 and Δ GK could bind to Kir3.2c (Figure 2B; data not shown), it was rather a surprise that neither of them could sensitize the Kir3.2c channel expressed in *Xenopus* oocytes (Figure 2A). This suggests the possibility that the GK domain might be involved in the SAP97-induced sensitization of Kir3.2c to G protein stimulation. Thus, we examined the effects of different overexpressed parts of SAP97 on the wild-type SAP97-induced sensitization of Kir3.2c (Figure 2C). Overexpression of Δ PDZ-123, which was composed of SH3 and GK but did not contain PDZ domains, almost completely prevented ACh induction of Kir3.2c current even in the presence of wild-type SAP97 ($0.17 \pm 0.07 \mu\text{A}$, $n = 3$) (Figure 2C). Overexpression of only the GK domain also completely suppressed ACh induction, while that of the SH3 domain had no inhibitory effect. These results suggest that the GK domain plays an indispensable role in the SAP97-induced sensitization of Kir3.2c to G protein stimulation, even though we could not detect any physical binding between the GK domain and Kir3.2c under our experimental conditions.

The effects of SAPAP1 on SAP97-induced sensitization of Kir3.2c

SAPAPs/GKAP/DAP are the proteins expressed at the postsynaptic site that bind specifically to the GK domain of the PSD/SAP protein family (Kim *et al.*, 1997; Naisbitt *et al.*, 1997; Satoh *et al.*, 1997; Takeuchi *et al.*, 1997). To examine further the possible functional roles of the GK domain of SAP97, we examined the effect of SAPAP1 upon ACh induction of Kir3.2c channel current in the presence of wild-type SAP97 (Figure 3). In the oocytes coexpressing SAPAP1, we could see neither background nor ACh-induced Kir currents ($n = 5$). Thus, SAPAP1 clearly prevented the SAP97-induced sensitization of Kir3.2c to G protein, probably by binding to the GK domain, although it was reported that SAPAPs increase the recruitment of PSD-95 to the plasma membrane (Takeuchi *et al.*, 1997). This result further supports the notion that the GK domain of SAP97 plays a critical role in the sensitization of the Kir3.2c channel.

Discussion

The primary function of the PSD-95/SAP90 family of anchoring proteins is to cluster ion channels and receptors at postsynaptic sites. The PDZ domains of the anchoring proteins are mainly responsible for this function. The anchoring proteins also act as scaffolds by arranging signal molecules in a certain order that is determined by the specific binding of signal molecules to different PDZ domains. Recent studies have revealed that the GK domain of PSD/SAP family proteins, although it has no guanylate kinase activity (Kistner *et al.*, 1995), acts as a binding domain for SAPAPs/GKAP/PAP (Kim *et al.*, 1997; Satoh *et al.*, 1997; Takeuchi *et al.*, 1997), MAP1A (Brennan *et al.*, 1998), BEGAIN (Deguchi *et al.*, 1998) or the kainate receptor (Garcia *et al.*, 1998). However, the functional role of the GK domain has remained largely unclarified. We show here that a deaf K_G channel composed

Fig. 3. The effects of SAPAP1 on the muscarinic response of Kir3.2c recorded in the presence of SAP97. (A) Representative traces and I-V curve of Kir3.2c plus SAP97 and Kir3.2c, SAP97 plus SAPAP1. (B) Combinations of cRNAs of Kir3.2c alone, Kir3.2c plus SAPAP1, Kir3.2c plus SAP97, and Kir3.2c, SAP97 plus SAPAP1 were injected into oocytes with cRNA of M₂R, and their Ba²⁺ (1 mM)-sensitive Kir currents were recorded at -120 mV in the presence of 10 μM ACh. The vertical bars indicate the SE. cRNA of SAPAP1 was injected at a 4-fold higher concentration than that of SAP97.

of homomeric Kir3.2c only reacts to G protein stimulation when bound to SAP97, where the GK domain of SAP97 plays a critical role.

SAP97 might affect Kir3.2c channel activity either by recruiting the channels from the intracellular pool to the plasma membrane or by directly controlling the channel function. The first possibility is unlikely, because (i) in both GFP fluorescence and immunoelectron microscopic measurements, even when expressed alone, Kir3.2c seemed to be localized on the plasma membrane of *Xenopus* oocytes and its localization was unaffected by the coexpression of SAP97 (Inanobe *et al.*, 1999; see Figure 1); (ii) overexpression of the GK domain prevented the effect of wild-type SAP97 even though the GK domain itself does not bind to Kir3.2c; and (iii) SAPAP1, which binds specifically to the GK domain, prevented the effect of SAP97. Therefore, it may be reasonable to postulate that SAP97 by directly controlling channel function confers the G protein sensitivity to the Kir3.2c channel. Theoretically, the whole-cell channel current can be enhanced by increase of either functional channel number, individual channel open probability or unitary channel conductance. Although preliminary studies using the single channel recording

technique have suggested that the third possibility was unlikely (not shown), the first two possibilities were difficult to discriminate. Further detailed studies on the properties of the expressed channel currents are needed to clarify this point.

None of the Δ PDZ-1, Δ PDZ-12 and Δ PDZ-123 mutants sensitized Kir3.2c channels (Figure 2A). Because Kir3.2c may bind to the second PDZ domain, the channel may not bind to Δ PDZ-12 and Δ PDZ-123 mutants. The Δ PDZ-1 mutant, however, lacks only the first PDZ domain and the N-terminus of SAP97, and would interact with Kir3.2c. Because it was shown that the N-terminal end of SAP97 was mandatory for the sorting of the protein to the cell membrane in epithelial cells (Wu *et al.*, 1998), it is also possible that all three mutants including Δ PDZ-1 were not transported to the oocyte plasma membrane and could not interact with the channel. Neither PDZ-123 nor Δ GK mutants could sensitize Kir3.2c channels (Figure 2A), although these mutants possessed all three PDZ domains and the N-terminal end of SAP97. These results may indicate that the binding of the second PDZ domain of SAP97 and the C-terminal end of Kir3.2c is necessary but insufficient to sensitize the channel. The essential role of GK domain in this task is, therefore, obvious from the present results. It remains unclear, however, how the GK domain opens the door of Kir3.2c for the access of $G_{\beta\gamma}$. There may be several possibilities for this mechanism. For example, the GK domain plays an essential role in forming the structural arrangement of SAP97, which is needed for Kir3.2c sensitization to G proteins by SAP97. It was reported that the GK domain bound to SH3 but not any PDZ domains within PSD-95 as well as DLG molecules (Brenman *et al.*, 1998; McGee and Brecht, 1999). This intramolecular interaction between SH3 and GK domains may be a candidate for the mechanism of the structural arrangement of SAP97. However, it was shown that the overexpressed GK domain alone can not associate with either SH3-GK protein or full-length PSD-95. Because the SAP97-induced sensitization of Kir3.2c channels was prevented by coexpression of the GK domain (Figure 2C), the intramolecular interaction between the SH3 and GK domains can not explain the present results. However, it is still possible that weak association between the GK domain and the Kir3.2c channel or other parts of SAP97 may exist and cause Kir3.2c sensitization through the conformational change of Kir3.2c and/or SAP97. The second possibility is that the GK domain may interact with unidentified molecules intrinsically expressed in *Xenopus* oocytes, which is essential for SAP97-induced sensitization of Kir3.2c. During this series of experiments for >6 months, we noticed that in the oocytes from certain frogs the SAP97-induced sensitization of the Kir3.2c channel to G protein stimulation did not occur, while in those from most frogs we could clearly see the effect as described previously. This may suggest that the second possibility is likely. Further studies are required to clarify this point.

This study shows for the first time that PSD/SAP family anchoring proteins directly control the function of synaptic ion channels. This indicates a possibility that the ion channels so far reported as nonfunctional in the heterologous expression assay may be functional *in vivo* by associating with other molecules including MAGUKs. For

example, δ glutamate receptor subunit expressed alone could not form functional channels (Lomeli *et al.*, 1993). However, the glutamate receptor was shown to bind to PSD-93 (Roche *et al.*, 1999), and the effect of coexpression of the PDZ-anchoring protein on the subunit function should be examined. Because the function of SAP97 could be modulated by SAPAP1 (Figure 3), synaptic ion channels seem to be controlled by a network of intracellular proteins. SAPAPs are expressed differentially among various cultured hippocampal neurons (Rao *et al.*, 1998); therefore, they might be one physiological factor controlling the properties of K_G channels expressed in different neurons. This system could be a novel target for various cell signals to modulate synaptic transmission in the brain.

Materials and methods

Functional expression in *Xenopus* oocytes and electrophysiological measurements

Rat SAP97 and PSD-95 cDNAs were obtained by RT-PCR cloning from a rat brain cDNA library. The cDNA that we obtained was a splicing variant of rat SAP97 reported previously (Müller *et al.*, 1995), i.e. amino acids residues 161–194, 407–421 and 695–701 were deleted, and AFRKNH (312–317), S (406) and H (694) were substituted for PASEKIM, T and R, respectively. The truncated clone of Kir3.2c, Kir3.2c- Δ C4, was constructed using the GeneEditor™ *in vitro* site-directed mutagenesis system (Promega Corp., Madison, WI). Various types of deletion mutants of SAP97 were constructed with PCR methods (all mutants except for PDZ-123 and Δ GK) or the GeneEditor™ system (PDZ-123 and Δ GK) (Promega) and inserted into the pGEMHE vector as reported previously (Inanobe *et al.*, 1999). The pGEMHE vectors expressing the mutants of SAP97 contain the SAP97-cDNA sequence encoding the following amino acids: Δ PDZ-1, 282–857; Δ PDZ-12, 407–857; PDZ-123, 1–507; Δ GK, 1–621; Δ PDZ-123, 533–857; SH3, 533–621; GK, 653–857. The methods of preparation of oocytes, cRNA injection and electrophysiological measurements have been described previously (Sugimoto *et al.*, 1992; Inanobe *et al.*, 1999). Each cDNA transcript was obtained with the mRNA capping kit (Stratagene, La Jolla, CA). Fifty nanograms of each cRNA dissolved in 50 nl of sterile water were manually injected into defolliculated *Xenopus* oocytes. After injection, oocytes were incubated in a modified Bath's solution at 18°C, and electrophysiological studies were undertaken 72–96 h later.

Two-electrode voltage-clamp experiments were performed with a commercially available amplifier (model TEC 01C; Turbo Clamp, Tamm, Germany) with microelectrodes that had resistances of 0.5–1.5 M Ω when filled with 3 M KCl. Oocytes were bathed in a solution that contained (in mM) 90 KCl, 3 MgCl₂, 5 HEPES-KOH pH 7.4 and 150 μ M niflumic acid to block endogenous chloride current. Voltage steps (1.2 s in duration) from the holding potential of 0 mV to potentials between +60 and –120 mV with –20 mV increments were delivered every 3 s. Experiments were performed at room temperature (20–22°C). Electrophysiological data were stored on video tapes using a PCM data recording system (NF Electronic Design, Tokyo, Japan) and subsequently replayed for computer analysis (Patch Analyst Pro; MT Corporation, Hyogo, Japan). The data were expressed in means $\pm$ SE.

Antibodies

Affinity-purified anti-G2A-5 antibody, which was raised against the amino acid sequences in the C-terminus of Kir3.2a and Kir3.2c (WSVSSKLNQHALE), was prepared as reported previously (Inanobe *et al.*, 1999). A mouse monoclonal anti-dlg antibody, which specifically recognizes the mouse, rat and human dlg/SAP97, and an anti-GIRK2 antibody were obtained commercially (Transduction Laboratories, Lexington, KY and Alamone Laboratory, Jerusalem, Israel, respectively).

Histological analyses of Kir3.2 isoforms in *Xenopus* oocytes

GFP-tagged Kir3.2a and Kir3.2c were constructed as described previously (Inanobe *et al.*, 1999). Electrophysiological properties of these clones were identical to those of the wild type (not shown). The oocytes were fixed with 4% (w/v) paraformaldehyde (PFA) for 1 h at 4°C. The oocytes were cut into 20 μ m sections, mounted on glass slides with 10% (v/v) glycerol in phosphate-buffered saline (PBS) and examined with a confocal microscope (model MRC-1024; Bio-Rad, Hercules, CA).

Immunogold electron microscopy was performed as described previously (Gotow *et al.*, 1995). The oocytes were fixed with 4% PFA and 0.1% (w/v) glutaraldehyde overnight at 4°C and dehydrated in 2.3 M sucrose containing 0.1 M sodium phosphate pH 7.4, and frozen in liquid nitrogen. Cryothin sections were cut on a microtome equipped with cryo-attachment (OmU4, Reichert, Vienna, Austria) and collected on Formvar carbon-coated grids. The cryothin sections on grids were treated with 1% BSA in PBS and incubated with anti-GIRK2, and then goat anti-rabbit IgG coupled to 5 nm colloidal gold particles (Amersham Pharmacia Biotech., Uppsala, Sweden). The sections were again fixed with 2% glutaraldehyde and post-fixed with 1% OsO₄, stained with 0.5% uranyl acetate, dehydrated in ethanol and embedded in London Resin white.

Biochemical analysis

Fusion proteins with GST of specific regions of Kir3.2c, Kir3.2a and SAP97 were constructed by subcloning PCR-amplified DNA fragments directionally into the *EcoRI*–*EcoRI* or *Bam*HI–*EcoRI* sites of pGEX-2T (Amersham Pharmacia Biotech.). Vectors expressed GST-fusion proteins containing the following amino acids: GST–Kir3.2c-C, 192–425; GST–Kir3.2c-ΔC4-C, 192–421; GST–Kir3.2a-C, 192–414; GST–Kir3.2a/c-N, 1–96 in Kir3.2c; GST–APDZ-12, 407–857; GST–ΔPDZ-123, 533–857; GST–PDZ-1, 188–285; GST–PDZ-2, 282–421; GST–PDZ-3, 407–507; GST–PDZ-123, 188–507; GST–SH3, 533–621; GST–GK, 653–857 in SAP97. Each fusion protein was expressed in *Escherichia coli* and purified on glutathione–Sepharose. HEK293T cells transfected with Kir3.2c or SAP97 were homogenized and solubilized in a lysis buffer [40 mM Tris–HCl pH 7.4, 0.15 M NaCl, 10 mM EDTA, 0.2 mg/ml benzamide, 30 kallikrein inhibitory units of aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride, and 1% (w/v) Triton X-100] and then centrifuged at 100 000 g for 30 min at 4°C. Solubilized homogenates were incubated for 1 h with 2.5 μg of purified fusion protein bound to 20 μl of glutathione–Sepharose. Samples were washed four times with PBS containing 0.4 M NaCl, resolved by SDS–8.5% polyacrylamide gel electrophoresis (SDS–PAGE), and then analyzed by Western blotting using anti-G2A-5 or anti-dlg antibodies.

Acknowledgements

We thank Dr Ian Findlay (Tours, France) for his critical reading of this manuscript, Ms Mari Imanishi and Ms Kazue Takahashi for their technical assistance, and Ms Keiko Tsuji for secretarial work. This work was supported by the grants to Y.K. from the Ministry of Education, Culture, Sports and Science of Japan, from the Research for the Future Program of the Japan Society for the Promotion of Science (96L00302), and from the Human Frontier Science Program (RG0158/1997-B).

References

Brenman, J.E., Topinka, J.R., Cooper, E.C., McGee, A.W., Rosen, J., Milroy, T., Ralston, H.J. and Bredt, D.S. (1998) Localization of postsynaptic density-93 to dendritic microtubules and interaction with microtubule-associated protein 1A. *J. Neurosci.*, **18**, 8805–8813.

Craven, S.E. and Bredt, D.S. (1998) PDZ proteins organize synaptic signaling pathways. *Cell*, **93**, 495–498.

Deguchi, M., Hata, Y., Takeuchi, M., Ide, N., Hirao, K., Yao, I., Irie, M., Toyoda, A. and Takai, Y. (1998) BEGAIN (brain-enriched guanylate kinase-associated protein), a novel neuronal PSD-95/SAP90-binding protein. *J. Biol. Chem.*, **273**, 26269–26272.

Froehner, S.C. (1993) Regulation of ion channel distribution at synapses. *Annu. Rev. Neurosci.*, **16**, 347–368.

Garcia, E.P., Mehta, S., Blair, L.A.C., Wells, D.G., Shang, J., Fukushima, T., Fallon, J.R., Garner, C.C. and Marshall, J. (1998) SAP90 binds and clusters kainate receptors causing incomplete desensitization. *Neuron*, **21**, 727–739.

Gee, S.H., Madhavan, R., Levinson, S.R., Caldwell, J.H., Sealock, R. and Froehner, S.C. (1998) Interaction of muscle and brain sodium channels with multiple members of the syntrophin family of dystrophin-associated proteins. *J. Neurosci.*, **18**, 128–137.

Gotow, T., Tanaka, J. and Takeda, M. (1995) The organization of neurofilaments accumulated in perikaryon following aluminium administration: relationship between structure and phosphorylation of neurofilaments. *Neuroscience*, **64**, 553–569.

Hall, Z. and Sanes, J.R. (1993) Synaptic structure and development: the neuromuscular junction. *Cell*, **72** (Suppl.), 99–121.

Inanobe, A. *et al.* (1999) Characterization of G-protein-gated K⁺ channels

composed of Kir3.2 subunits in dopaminergic neurons of the substantia nigra. *J. Neurosci.*, **19**, 1006–1017.

Kim, E., Naisbitt, S., Hsueh, Y.-P., Rao, A., Rothschild, A., Craig, A.M. and Sheng, M. (1997) GKAP, a novel synaptic protein that interacts with the guanylate kinase-like domain of the PSD-95/SAP90 family of channel clustering molecules. *J. Cell Biol.*, **136**, 669–678.

Kistner, U., Garner, C.C. and Linial, M. (1995) Nucleotide binding by the synapse associated protein SAP90. *FEBS Lett.*, **359**, 159–163.

Lomeli, H., Sprengel, R., Laurie, D.J., Köhr, G., Herb, A., Seeburg, P.H. and Wisden, W. (1993) The rat δ1 and δ2 subunits extend the excitatory amino acid receptor family. *FEBS Lett.*, **315**, 318–322.

Lüscher, C., Jan, L.Y., Stoffel, M., Malenka, R.C. and Nicoll, R.A. (1997) G protein-coupled inwardly rectifying K⁺ channels (GIRKs) mediate postsynaptic but not presynaptic transmitter actions in hippocampal neurons. *Neuron*, **19**, 687–695.

McGee, A.W. and Bredt, D.S. (1999) Identification of an intramolecular interaction between the SH3 and guanylate kinase domains of PSD-95. *J. Biol. Chem.*, **274**, 17431–17436.

Müller, B.M., Kistner, U., Veh, R.W., Cases-Langhoff, C., Becker, B., Gundelfinger, E.D. and Garner, C.C. (1995) SAP97, a novel member of the super family of brain-related guanylate kinases. *J. Neurosci.*, **15**, 2354–2366.

Naisbitt, S., Kim, E., Weinberg, R.J., Rao, A., Yang, F.-C., Craig, A.M. and Sheng, M. (1997) Characterization of guanylate kinase-associated protein, a postsynaptic density protein at excitatory synapses that interacts directly with postsynaptic density-95/synapse-associated protein 90. *J. Neurosci.*, **17**, 5687–5696.

Rao, A., Kim, E., Sheng, M. and Craig, A.M. (1998) Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. *J. Neurosci.*, **18**, 1217–1229.

Roche, K.W., Dune Ly, C., Petralia, R.S., Wang, Y.-X., McGee, A.W., Bredt, D.S. and Wenthold, R.J. (1999) Postsynaptic density-93 interacts with the δ2 glutamate receptor subunit at parallel fiber synapse. *J. Neurosci.*, **19**, 3926–3934.

Saras, A. and Heldin, C.H. (1996) PDZ domains bind carboxy-terminal sequences of target proteins. *Trends Biochem. Sci.*, **21**, 455–458.

Sato, K. *et al.* (1997) DAP-1, a novel protein that interacts with the guanylate kinase-like domains of hDLG and PSD-95. *Genes Cells*, **2**, 415–424.

Sheng, M. (1996) PDZs and receptor/channel clustering: rounding up the latest suspects. *Neuron*, **17**, 575–578.

Sugimoto, T., Tsuchimochi, H., McGregor, C.G.A., Mutoh, H., Shimizu, T. and Kurachi, Y. (1992) Molecular cloning and characterization of the platelet-activating factor receptor gene expressed in the human heart. *Biochem. Biophys. Res. Commun.*, **189**, 617–624.

Takeuchi, M., Hata, Y., Hirao, K., Toyoda, A., Irie, M. and Takai, Y. (1997) SAPAPs. A family of PSD-95/SAP90-associated proteins localized at postsynaptic density. *J. Biol. Chem.*, **272**, 11943–11951.

Wu, H., Reuver, S.M., Kuhlendahl, S., Chung, W.J. and Garner, C.C. (1998) Subcellular targeting and cytoskeletal attachment of SAP97 to the epithelial lateral membrane. *J. Cell Sci.*, **111**, 2365–2376.

Yamada, M., Inanobe, A. and Kurachi, Y. (1998) G protein regulation of potassium ion channels. *Pharmacol. Rev.*, **50**, 723–757.

Ziff, E.B. (1997) Enlightening the postsynaptic density. *Neuron*, **19**, 1163–1174.

Received October 5, 1999; revised November 8, 1999;
accepted November 10, 1999