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Activation of channel activity of the NMDA receptor-PSD-95 complex by guanylate kinase-associated protein (GKAP)

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Abstract **The channel-associated protein PSD-95 functionally modulates NMDA receptor channels, interacting with the channels via PDZ domain of PSD-95. PSD-95 also interacts with guanylate kinase-associated protein (GKAP) through the guanylate kinase-like domain of PSD-95. Here we report that GKAP markedly potentiates the channel activity of the receptor-PSD-95 complex. However, GKAP had no effect on basic properties of the channels nor on PSD-95-induced changes in channel properties. Thus, GKAP affects the channel activity of the NMDA receptor via PSD-95 quantitatively, which may make signal transmission more efficient at postsynaptic sites.**

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Key words: *N*-Methyl-D-aspartate receptor; Guanylate kinase-associated protein; PSD-95; Channel activity; *Xenopus* oocyte

1. Introduction

The *N*-methyl-D-aspartate (NMDA) receptor is a subclass of ionotropic glutamate receptors in the mammalian brain, and exists as heteromultimers of the ζ1 subunit (NR1) with ε subunits (NR2s) [1,2]. Different combinations of these subunits exhibit distinct channel properties and characteristic regional and developmental expression in vivo [3–5]. The activation of NMDA receptors is essential for induction of synaptic long-term potentiation (LTP) which underlies the formation and storage of some forms of memory [6].

The ε subunits of the NMDA receptor have been shown to interact with PSD-95 [7,8]. PSD-95 and its family proteins are localized at postsynaptic density in neuronal cells, and are characterized by the presence of three PDZ domains in the NH_2-terminal region, followed by SH3 and guanylate kinase-like (GK) domains [9–15]. The first and second PDZ domains interact with the COOH-terminal E-T/S-X-V sequence motif of NMDA receptor ε subunits [7,8] and of K^+ channels [16,17]. The interaction of these proteins induces the clustering of the channel proteins [14,16,18]. A protein interacting with the GK domain of PSD-95 has been identified by the yeast two-hybrid method, and named GKAP (guanylate kinase-associated protein) or SAPAP (SAP90/PSD-95-associated protein) [19–21]. GKAP co-localizes with PSD-95 in hippocampal neurons [20,22], and forms a complex with the NMDA receptor and PSD-95 [19,20].

Recently, we found that PSD-95 functionally modulates the ε2/ζ1 heteromeric NMDA receptor channels; it decreases the affinity of the channels to L-glutamate and inhibits the protein kinase C-mediated potentiation of the channels [23]. In this study, we examined whether the channel activity of the receptor-PSD-95 complex is further modulated by GKAP, and found that GKAP significantly potentiates the channel activity of the NMDA receptor through PSD-95.

2. Materials and methods

2.1. Preparation of cRNA and oocytes

Complementary RNAs of NMDA receptors and PSD-95 were synthesized as described previously [23]. Deletion mutants of PSD-95 were prepared as described previously [23]. The cDNA fragment of GKAP (nucleotide positions 19–2343, GenBank U67987) was subcloned into pBKSA [24]. Complementary RNA of GKAP was synthesized in vitro with T3 RNA polymerase using the *Not*I-cleaved plasmid as a template.

Stage V and VI oocytes were obtained from *Xenopus laevis* as described previously [23]. The oocytes were injected with ε2 and ζ1 cRNAs in a molar ratio of 1:2 (ε2:ζ1). The total amounts of cRNA injected were 5 ng per oocyte. For co-expression of PSD-95, the synthesized PSD-95 cRNA (12.5 ng) was injected 24 h after the injection of the NMDA receptor cRNAs. For co-expression of PSD-95 and GKAP, cRNAs of PSD-95 (12.5 ng) and GKAP (12.5–25 ng) were co-injected. Before recording, oocytes were incubated at 19°C for 18–26 h in Barth's medium (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, and 7.7 mM Tris-HCl, pH 7.2).

2.2. Electrophysiological recordings

Currents were recorded with two-electrode voltage-clamp techniques using a CA-1a high performance oocyte clamp (Dagan Corp., Minneapolis, MN, USA). Electrodes were filled with 3 M KCl and had resistances of 1–5 MΩ. Oocytes were perfused by a constant stream of Ba^{2+} Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $BaCl_2$, and 10 mM HEPES, pH 7.2) at 23–25°C. The oocyte membrane was voltage-clamped at −70 mV. In the standard assay, currents were evoked by bath perfusion of Ba^{2+} Ringer solution containing 100 μM L-glutamate and 10 μM glycine for 20 s, followed by a washout with standard Ba^{2+} Ringer solution. Current signals were digitized for analysis, statistical significance was determined by ANOVA followed by Dunnett's multiple comparison test. $P < 0.05$ was considered to be statistically significant.

2.3. Immunoblotting

Polyclonal antibodies against ε2 and ζ1 subunits of NMDA receptor were obtained from Calbiochem (La Jolla, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. After measurement of current responses, oocytes were combined and homogenized with 100 mM NaCl, 5 mM EDTA, 20 mM Tris-HCl, pH 7.5, and then centrifuged at $1000\times g$ for 10 min. The supernatant was centrifuged at $200\,000\times g$ for 30 min. The obtained pellet (total membrane fraction) was solubilized with Laemmli gel sample buffer. The samples were separated by SDS-polyacrylamide gel electrophoresis on a 5% gel, and transferred to a nitrocellulose sheet. After blocking with 5% skim milk and 0.05% Tween 20 in Tris-HCl-buffered saline, the sheets were incubated with primary antibodies in block solution. Labeled bands were visualized using enhanced chemiluminescence (Pierce, Rockford, IL, USA). The bands were analyzed by densitometric scanning using Densitograph AE-6900M (Atto, Tokyo, Japan). The

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amounts of the proteins were quantified from the intensity of the bands, which has a linearity to the amounts of the samples applied to the gel.

3. Results and discussion

3.1. Co-expression of NMDA receptor channels, PSD-95 and GKAP

To investigate the effects of GKAP (or SAPAP1) on the ε2/ζ1 heteromeric NMDA receptor-PSD-95 complex, we expressed the NMDA receptor, PSD-95 and GKAP in *Xenopus* oocytes by injection of in vitro synthesized cRNAs, and measured current responses to 100 μM L-glutamate and 10 μM glycine in Ba^{2+} Ringer solution, comparing them with those in oocytes expressing the receptor and co-expressing PSD-95. Typical traces of the current responses are shown in Fig. 1. The average of the current responses from about 50 oocytes was 2-fold increased by expression of PSD-95, and was 5-fold increased by co-expression of PSD-95 and GKAP (Table 1). Since co-expression of PSD-95 and/or GKAP may change the expression level of the receptor, we compared the amounts of the receptor expressed in these oocytes by immunoblotting. In accord with our previous results [23], PSD-95 caused a 2-fold increase in the expression of the receptor, so that the channel activity normalized by the expression level of the receptor was almost the same as that in oocytes expressing the receptor alone (Table 1). On the other hand, co-expression of PSD-95 and GKAP caused a 1.7-fold increase in the expression of the receptor (Table 1). The normalized channel activity was about 3-fold increased in these oocytes (Table 1). This potentiation is not due to a direct effect of GKAP on the receptor, because no difference in the channel activity was observed between oocytes expressing the receptor alone and co-expressing GKAP without PSD-95 (Fig. 2) and because the potentiation of the channel activity was not observed in oocytes co-expressing the receptor, GKAP and the deletion mutant of PSD-95 which lacks GK domain (Fig. 2). The expression level of PSD-95 was not changed by co-expression of GKAP, when examined by the immunoblotting with anti-PSD-95 antibodies (data not shown). The immunoblotting also revealed that the ratio between ε2 and ζ1 subunits was not changed among three groups of oocytes (Table 1), indicating that the PSD-95-GKAP complex does not inhibit plasma membrane insertion of the ε2 subunit.

3.2. Effects of GKAP on the channel properties of the NMDA receptor-PSD-95 complex

Recently, we reported that PSD-95 has two inhibitory effects on the ε2/ζ1 NMDA receptor; PSD-95 decreases the sensitivity of the channels to L-glutamate, and inhibits the protein kinase C-mediated potentiation of the channels [23]. We next examined whether GKAP changes these channel properties of the receptor interacting with PSD-95. As we reported previously [23], PSD-95 decreased the sensitivity of the NMDA receptor channels to L-glutamate, shifting the dose-response curve to the right (Fig. 3). GKAP did not change the lower sensitivity of the channels to L-glutamate, which was caused by PSD-95 (Fig. 3). The channel activity of the ε2/ζ1 NMDA receptor is markedly potentiated by treatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) [25–27]. PSD-95 inhibits the potentiation of the channels induced by TPA (Fig. 4) [23]. The potentiation by TPA was not observed in oocytes co-expressing PSD-95 and GKAP (Fig. 4). The latter observation indicates that the binding of GKAP to PSD-95 does not affect the inhibition of protein kinase C-mediated channel potentiation, which is caused by PSD-95. However, the possibility cannot be ruled out that the interaction between PSD-95 and GKAP is disrupted by protein kinase C-catalyzed phosphorylation. There was no difference in the glycine sensitivity, the current-voltage relationship and a voltage-dependent Mg^{2+} block of the channels among three groups of oocytes expressing the receptor alone, co-expressing PSD-95, and co-expressing PSD-95 and GKAP (data not

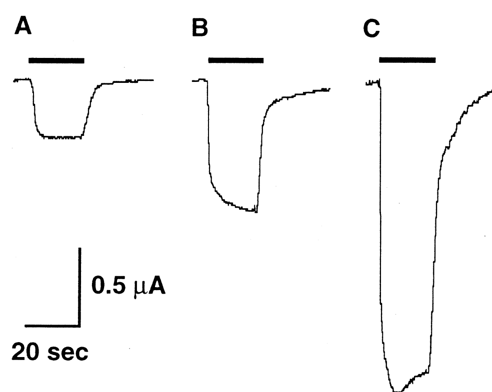

Fig. 1. Current responses to L-glutamate in oocytes co-expressing the ε2/ζ1 heteromeric NMDA receptor, PSD-95 and GKAP. Oocytes were injected with in vitro synthesized cRNAs of the NMDA receptor (A), of the receptor and PSD-95 (B), or of the receptor, PSD-95 and GKAP (C). Current responses were measured in Mg^{2+}-free Ba^{2+} Ringer solution at −70 mV membrane potential as described in Section 2. Bars show the duration of application of 100 μM L-glutamate with 10 μM glycine. Inward current is downward.

Table 1
Channel activity and amounts of expressed NMDA receptor subunits

Oocyte	Current response (μA)	Amounts of ε2 (arbitrary units)	Amounts of ζ1 (arbitrary units)	ε2/ζ1	Normalized channel activity (current response/ε2) (μA/arbitrary unit)
ε2/ζ1	0.345 ± 0.025 (55)	0.735	1.570	0.468	0.469
ε2/ζ1+PSD-95	0.757 ± 0.043* (57)	1.414	2.826	0.500	0.535
ε2/ζ1+PSD-95+GKAP	1.812 ± 0.118*†	1.235	2.539	0.486	1.467

Current responses evoked by 100 μM L-glutamate and 10 μM glycine were measured in the number of oocytes indicated in parentheses, and are presented as mean ± S.E.M. After measurement of current responses, oocytes of each group were combined and the total membrane fraction was prepared. The samples were separated by SDS-polyacrylamide gel electrophoresis on a 5% gel, and immunoblotting was performed with anti-ζ1 subunit and anti-ε2 subunit antibodies as described in Section 2.
*$P < 0.001$ compared with oocytes expressing the NMDA receptor (ε2/ζ1) alone; †$P < 0.001$ compared with oocytes expressing the NMDA receptor and PSD-95.

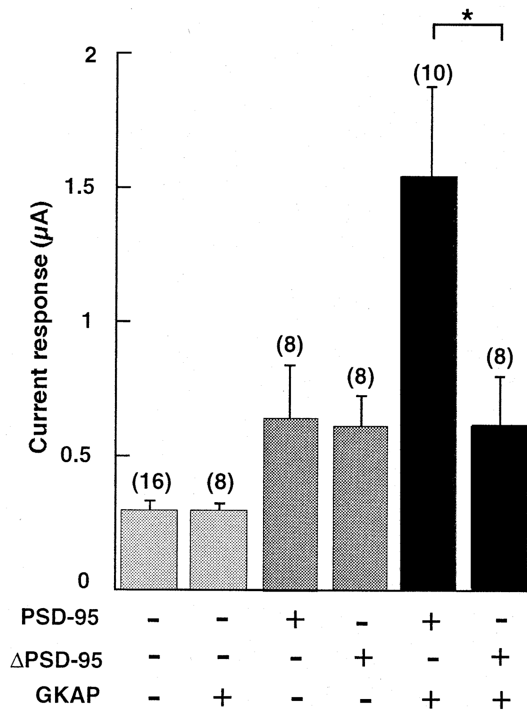

Fig. 2. Current responses to L-glutamate in oocytes expressing the NMDA receptor with GKAP and/or deletion mutant of PSD-95. Oocytes were injected with various combinations of cRNAs of the NMDA receptor, PSD-95 or deletion mutant of PSD-95 (ΔPSD-95), and GKAP. Current responses to 100 μM L-glutamate with 10 μM glycine were measured. The deletion mutant which lacks SH3 and GK domains had the same effects on the NMDA receptor channels as the wild-type PSD-95, as reported previously [23]. However, the potentiation of current responses by GKAP was not observed with this mutant. The data shown are the mean ± S.E.M. of the number of oocytes indicated in parentheses. $^{*}P < 0.01$.

shown). Thus, GKAP does not change the channel properties of the NMDA receptor-PSD-95 complex, although it markedly potentiates the channel activity of the receptor in the presence of PSD-95.

3.3. Possible roles of GKAP in modulation of NMDA receptor channels

We demonstrated that GKAP potentiates the channel activity of the NMDA receptor via PSD-95, while it does not affect the inhibitory effects of PSD-95 on the channels. What is the functional significance of these two modes of the channel modulation? The inhibitory effects of PSD-95 on the NMDA receptor channels might be related to induction of LTP. It is known that protein kinase C is necessary for induction of LTP [6]. PSD-95 may elevate the threshold of LTP induction, inhibiting the protein kinase C-mediated potentiation of the NMDA receptor channels. It was reported that LTP is enhanced in mutant mice lacking PSD-95 [28]. Based on neural network models incorporating bidirectional synaptic plasticity (potentiation and depression), the enhanced LTP which can be induced by low-frequency stimulation is accompanied by severely impaired learning and memory. In those mutant mice, in fact, spatial learning was impaired [28]. On the other hand, GKAP increases the channel activity. Since GKAP did not alter the basic channel properties nor a PSD-95-induced change in channel properties, GKAP may increase

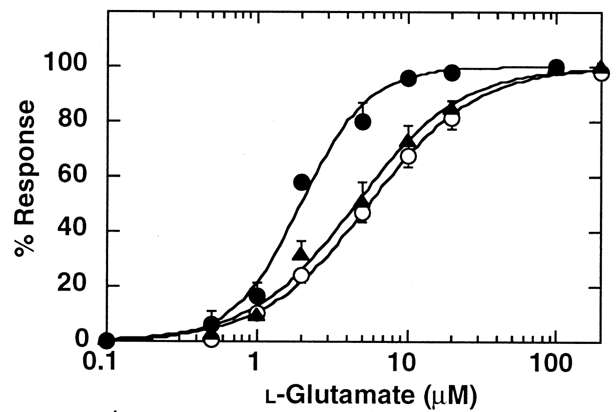

Fig. 3. Dose-response curves for L-glutamate in oocytes co-expressing the ε2/ζ1 NMDA receptor, PSD-95 and GKAP. Various concentrations of L-glutamate with 10 μM glycine were applied, and the steady-state currents were measured in oocytes injected with the NMDA receptor cRNAs (closed circles), with the receptor and PSD-95 cRNAs (open circles), or with the receptor, PSD-95 and GKAP cRNAs (closed triangles). Each point represents the mean ± S.E.M. of the current amplitudes obtained from five oocytes. The EC_{50} values were 1.81 μM, 5.62 μM and 4.67 μM for oocytes expressing the NMDA receptor, co-expressing PSD-95, and co-expressing PSD-95 and GKAP, respectively, and the Hill coefficient values were 1.53, 1.02 and 1.16, respectively.

the number of functional channels, which is probably related to clustering of the channels. It is known that PSD-95 can induce the channel clustering without GKAP [14,16,18]. GKAP may strengthen the cluster scaffold. Thus, GKAP affects the channel activity of the NMDA receptor via PSD-95 quantitatively, which may make signal transmission more ef-

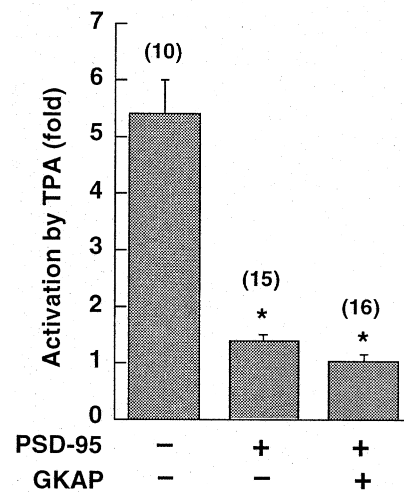

Fig. 4. Effects of TPA on current responses in oocytes co-expressing the ε2/ζ1 NMDA receptor, PSD-95 and GKAP. Current responses were measured in oocytes expressing the receptor, co-expressing PSD-95, and co-expressing PSD-95 and GKAP before and after bath application of 1 μM TPA for 10 min. The potentiation of current response by TPA treatment is presented. The data shown are the mean ± S.E.M. of the number of oocytes indicated in parentheses. The potentiation of current response by TPA was 5.43 ± 0.75, 1.41 ± 0.10, and 1.06 ± 0.10 in oocytes expressing the receptor, co-expressing PSD-95, and co-expressing PSD-95 and GKAP, respectively. $^{*}P < 0.01$ compared with oocytes expressing the receptor alone. There was no statistical difference in the potentiation between oocytes co-expressing PSD-95, and co-expressing PSD-95 and GKAP.

ficient at postsynaptic sites. Further studies are necessary to elucidate the mechanism of the channel potentiation by GKAP via PSD-95.

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