Modulation of the Channel Activity of the $\epsilon_2/\zeta_1$-Subtype N-Methyl d-Aspartate Receptor by PSD-95*

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A channel-associated protein PSD-95 has been shown to induce clustering of N-methyl d-aspartate (NMDA) receptors, interacting with the COOH terminus of the $\epsilon$ subunit of the receptors. The effects of PSD-95 on the channel activity of the $\epsilon_2/\zeta_1$ heteromeric NMDA receptor were examined by injection of PSD-95 cRNA into Xenopus oocytes expressing the NMDA receptors. Expression of PSD-95 decreased the sensitivity of the NMDA receptor channels to L-glutamate. Mutual studies showed that the interaction between the COOH terminus of the $\epsilon_2$ subunit of the NMDA receptor and the second PSD-95/Dlg/ZO-1 domain of PSD-95 is critical for the decrease in glutamate sensitivity. It is known that protein kinase C markedly potentiates the channel activity of the NMDA receptor expressed in oocytes. PSD-95 inhibited the protein kinase C-mediated potentiation of the channels. Thus, we demonstrated that PSD-95 functionally modulates the channel activity of the $\epsilon_2/\zeta_1$ NMDA receptor. PSD-95 makes signal transmission more efficient by clustering the channels at postsynaptic sites. In addition to this, our results suggest that PSD-95 plays a protective role against neuronal excitotoxicity by decreasing the glutamate sensitivity of the channels and by inhibiting the protein kinase C-mediated potentiation of the channels.

The NMDA$^1$ receptor is a subclass of ionotropic glutamate receptors in the mammalian brain. The NMDA receptors exhibit several channel properties distinct from other non-NMDA glutamate receptors, including a high Ca$^{2+}$/Na$^+$ permeability ratio, a voltage-dependent Mg$^{2+}$ block, and a requirement for glycine as co-agonist (1–4). The receptors are modulated by phosphorylation catalyzed by protein kinase C (5, 6) and by tyrosine kinases (7, 8). In brain, functional NMDA receptors are thought to exist as heteromultimers of the $\zeta_1$ subunit (NR1) with $\epsilon$ subunits (NR2 subunits). Different combinations of these subunits exhibit distinct channel properties and characteristic regional and developmental expression in vivo (9–11). Disruption of the $\zeta_1$ gene is lethal in mice (12), whereas that of the $\epsilon_1$ and $\epsilon_2$ genes results in reduced channel activity and reduced LTP in the hippocampus (13, 14). A number of evidences indicate that the activation of NMDA receptors is essential for induction of LTP, which underlies the formation and storage of some forms of memories (15).

NMDA receptors, interacting with the COOH terminus of the $\epsilon_2$ subunit of the NMDA receptor and the second PSD-95/Dlg/ZO-1 domain of PSD-95 is critical for the decrease in glutamate sensitivity. It is known that protein kinase C markedly potentiates the channel activity of the NMDA receptor expressed in oocytes. PSD-95 inhibited the protein kinase C-mediated potentiation of the channels. Thus, we demonstrated that PSD-95 functionally modulates the channel activity of the $\epsilon_2/\zeta_1$ NMDA receptor. PSD-95 makes signal transmission more efficient by clustering the channels at postsynaptic sites. In addition to this, our results suggest that PSD-95 plays a protective role against neuronal excitotoxicity by decreasing the glutamate sensitivity of the channels and by inhibiting the protein kinase C-mediated potentiation of the channels.

EXPERIMENTAL PROCEDURES

Preparation of cRNA and Oocytes—Plasmids pBKSA$^1$, pBKSA$^2$, and pBKSA$^1$ containing cDNAs encoding mouse brain $\epsilon_1$, $\epsilon_2$, and $\zeta_1$ subunits of the NMDA receptor, respectively, were kindly provided by Dr. Masayoshi Mishina (6, 28). Complementary RNAs of NMDA receptors were synthesized in vitro with T3 RNA polymerase using NotI-cleaved plasmids as templates. The coding region of PSD-95 was amplified by polymerase chain reaction from a rat brain cDNA library. The resulting product was subcloned into pSP64 poly(A) vector (Promega, Madison, WI). The construction was confirmed by dideoxynucleotide sequencing. The PSD-95 cDNA was synthesized in vitro with SP6 RNA polymerase using EcoRI-cleaved plasmid. Deletion and site-specific mutants of the NMDA receptor $\epsilon_2$ subunit and PSD-95 were constructed by polymerase chain reaction strategy using Pfu polymerase (Stratagene, La Jolla, CA) (see Fig. 7A). The wild-type $\epsilon_2$ subunit and PSD-95 were used as polymerase chain reaction templates for mutation, except the C(3,5)-cGMP(1–2) construct, for which the APD(1–2) construct was used as a template. Mutations were verified by dideoxynucleotide sequencing.

Stage V and VI oocytes were obtained from anesthetized Xenopus laevis and incubated for 2 h at room temperature with 2 mg/ml collagenase 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) containing 18 units/ml penicillin and 18 µg/ml streptomycin without Ca$^{2+}$. The follicular cell layer was then removed with forceps. The oocytes were injected with $\epsilon_2$ and $\zeta_1$ cRNAs at a molar ratio of 1:2. The total amounts of cRNA injected were 5 ng/oocyte. For co-expression of PSD-95, the synthesized PSD-95 cRNA (1–25 ng) was injected 24 h
after injection of the NMDA receptor cRNAs. Before recording, oocytes were incubated at 19 °C for 18–26 h in Barth’s medium.

**Electrophysiological Recordings**—Currents were recorded with two-electrode voltage-clamp techniques using a CA-1a high performance oocyte clamp (Dagan Corp., Minneapolis, MN). Electrodes were filled with 3 M KCl and had resistances of 1–5 megohms. Oocytes were perfused by a constant stream of BaCl₂ Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM BaCl₂, 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 BaCl₂ Ringer solution containing 100 μM L-glutamate and 10 μM glycine for 20 s, followed by a washout with standard BaCl₂ Ringer solution. Current signals were digitized for analyses; statistical significance was determined using Student’s t test.

**Immunoblotting**—Polyclonal antibodies against the NMDA receptor ε2 and 1 subunits were obtained from Calbiochem and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Monoclonal antibody against the central region (amino acids 353–504) of rat PSD-95 was obtained from Transduction Laboratories (Lexington, KY). Polyclonal antibodies against PSD-95 were raised in rabbits using a glutathione S-transferase fusion protein with the NH2-terminal region (amino acids 4–404) of rat PSD-95 as immunogen and were affinity-purified. For immunoblotting of PSD-95, oocytes were solubilized with Laemmli gel sample buffer containing 2% SDS (50 μg/oocyte) (29). For immunoblotting of the NMDA receptor, the total membrane fraction was prepared from oocytes. After measurement of current responses, oocytes were homogenized with 100 mM NaCl, 5 mM EDTA, and 20 mM Tris-HCl, pH 7.5, and then centrifuged at 1000 g × g for 10 min. The supernatant was centrifuged at 200,000 g × 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 5 or 7.5% gels and transferred to nitrocellulose sheets. After blocking with 5% skim milk and 0.05% Tween 20 in Tris-HCl-buffered saline, the sheets were incubated with primary antibodies in the blocking solution. Labeled bands were visualized using enhanced chemiluminescence (Pierce). The bands were analyzed by densitometric scanning using Densitograph AE-6900M (Atto Corp., Tokyo, Japan). The 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.

**RESULTS**

**Characterization of ε2/1 Heteromic NMDA Receptor Channels in Oocytes Co-expressing PSD-95**—To investigate the effects of PSD-95 on the channel activities of the ε2 (NR2B)/1 (NR1a) heteromic NMDA receptor, we expressed the NMDA receptor and PSD-95 in Xenopus oocytes by injection of in vitro synthesized cRNAs. When the expression of the proteins was monitored by immunoblotting, the level of the NMDA receptor reached a plateau within 24 h after injection and then decreased (data not shown). On the other hand, the level of PSD-95 rapidly increased to the maximum within 24 h after injection and then decreased (data not shown). Therefore, in this study, PSD-95 cRNA was injected into oocytes that were already expressing the NMDA receptor, and the effects of PSD-95 on the NMDA receptor channels were examined 18–26 h after injection of PSD-95 cRNA, measuring current responses to 100 μM L-glutamate and 10 μM glycine in BaCl₂ Ringer solution under voltage clamp at −70 mV. However, the results obtained with these oocytes were qualitatively the same as those obtained with oocytes in which cRNAs of the NMDA receptor and PSD-95 were coinjected.

To characterize the channels in oocytes co-expressing PSD-95, we first examined the effects of ifenprodil on the channel activity. Ifenprodil is known to selectively block ε2/1 heteromic NMDA receptor channels (30). Ifenprodil completely inhibited the channels in oocytes co-expressing the receptor and PSD-95, as in oocytes expressing the receptor alone, whereas it had no effect on other subtypes of NMDA receptor channels such as the ε1/1 heteromic receptor (Fig. 1). These results indicate that the current responses in oocytes co-expressing the receptor with PSD-95 are indeed through ε2/1 NMDA receptor channels. The average of the current responses from 15 oocytes was 1.34 ± 0.56 μA (mean ± S.D., n = 15) in oocytes expressing the NMDA receptor and 2.56 ± 1.00 μA (mean ± S.D., n = 15) in oocytes co-expressing PSD-95. When the expression of the receptor was compared in these oocytes by immunoblotting, the expression level of the receptor was proportional to the current response, and the ratio between ε2 and 1 subunits was not changed by PSD-95 (Fig. 2). These results indicate that PSD-95 does not inhibit the expression or plasma membrane insertion of the receptor. A higher expression level of the receptor in oocytes co-expressing PSD-95 might be due to the stabilization of the receptor by interacting with PSD-95. However, the amplitudes of evoked currents varied from oocyte to oocyte and could not simply be compared between oocytes expressing the receptor and co-expressing PSD-95. In this study, we focused on the qualitative differences in the channel properties between them. When the channel activities were measured at various concentrations of glutamate, the dose-response curve shifted to the right in oocytes co-expressing the receptor and PSD-95 (Fig. 3). The EC50 was increased from 1.39 to 5.76 μM by injection of PSD-95 cRNA. Thus, PSD-95 significantly decreased the sensitivity of the channels to glutamate. On the other hand, PSD-95 did not change other basic properties of the NMDA receptor such as a requirement for glycine as co-agonist, the current-voltage relationship, and a voltage-dependent Mg2⁺ block of the channels (data not shown).

**Effects of PSD-95 on the Protein Kinase C-mediated Potentiation of the Channels**—It was reported that the channel activity of the ε2/1 NMDA receptor in oocytes is markedly potentiated by treatment with TPA, due to activation of protein kinase C (28, 31, 32). Under our assay conditions, the evoked currents were 4-fold increased by 1 μM TPA in oocytes expressing the NMDA receptor (Figs. 4 and 5). In oocytes co-expressing PSD-95, however, little potentiation of the channel activity (1.3-fold) was observed with TPA treatment (Figs. 4 and 5). This inhibitory effect of PSD-95 on the potentiation of the channels by TPA was dependent on the amounts of PSD-95 cRNA injected into an oocyte (Fig. 5). To rule out the possibility that PSD-95 directly modulates protein kinase C in oocytes, we...
examined the effects of PSD-95 on the metabotropic glutamate receptor. The activity of metabotropic glutamate receptors in oocytes can be estimated by the channel activity of Ca"²⁺-activated chloride channels. When expressed in oocytes, metabotropic glutamate receptor-5 showed pronounced desensitization in response to an activation by 1 mM glutamate (data not shown), as reported previously (33). This desensitization has been shown to require protein kinase C-catalyzed phosphorylation of the receptor (33). Indeed, a brief treatment with TPA after activation by glutamate prolonged the desensitization. Co-expression of PSD-95 had no effect on the desensitization upon activation by 1 mM glutamate or on the prolonged desensitization by TPA (data not shown), indicating that expression of PSD-95 does not change the activity of protein kinase C in oocytes.

Analyses of the Channel Modulation by PSD-95 Using the Deletion Mutant of the NMDA Receptor—PSD-95 has been shown to interact with the COOH terminus of the NMDA receptor e2 subunit through the first two PDZ domains (18, 19, 25). To clarify a role of this interaction in the modulation of glutamate sensitivity, the effects of PSD-95 were examined on the mutant NMDA receptor that lacks the COOH-terminal four amino acid residues of the e2 subunit. The change in the sensitivity to glutamate was monitored by the ratio of current response at 100 μM glutamate to that at 10 μM. In accord with the dose-response curves (Fig. 3), expression of PSD-95 increased the ratio from 1 to −1.5 in oocytes expressing the wild-type NMDA receptor (Fig. 6). In oocytes expressing the mutant receptors, however, the ratio remained −1 regardless of PSD-95 expression (Fig. 6). These data indicate that the interaction of the COOH-terminal region of the e2 subunit with the PDZ domain of PSD-95 is essential for a decrease in sensitivity to glutamate.

Analyses of the Channel Modulation by PSD-95 Using Mutant PSD-95—The modulation of glutamate sensitivity by PSD-95 was further analyzed using a series of PSD-95 mutants (Fig. 7A). The expression of these constructs was compared with that of wild-type PSD-95 by immunoblotting with antibodies against the NH₂-terminal region (amino acids 4–404) and against the central region (amino acids 353–504) of rat PSD-95. When 25 ng of cRNA was injected into an oocyte for each mutant, there was no significant difference in the expres-
Modulation of NMDA Receptor Channel by PSD-95

In this study, we demonstrated that PSD-95 suppresses the protein kinase C-mediated potentiation of NMDA receptor channels (Figs. 4 and 5). It is unlikely that PSD-95 directly inhibits the kinase activity of protein kinase C in oocytes because PSD-95 had no effect on the desensitization of metabotropic glutamate receptor-5 in oocytes co-expressing both proteins, which was shown to require phosphorylation of metabotropic glutamate receptor-5 by protein kinase C (33).
Further study is necessary to elucidate how the NMDA receptor channels are potentiated by protein kinase C and how this potentiation is modulated by PSD-95.

Although \( \varepsilon_2/\zeta_1 \) NMDA channels in oocytes are potentiated by the treatments that activate protein kinase C (Fig. 4) (31, 32), it is still controversial whether phosphorylation by protein kinase C potentiates the NMDA receptor channels \textit{in vivo}. The protein kinase C-mediated potentiation of the channels is observed in dorsal horn neurons in spinal cord and caudal brain stem (5, 39), whereas activation of the kinase does not potentiate NMDA receptor-mediated responses in CA1 hippocampal neurons (40) where the \( \varepsilon_2 \) subunit is highly expressed (9, 10, 28). The latter observation can be explained in part by our finding that PSD-95 suppresses the protein kinase C-mediated potentiation of \( \varepsilon_2/\zeta_1 \) NMDA receptor channels.

The induction of LTP at synapses requires \( \text{Ca}^{2+} \) entry into the postsynaptic dendritic spine via NMDA receptors (15). LTP is triggered by delivering synchronous high frequency stimulation, a tetanus, to the pathway. In the widely accepted model, tetanic stimulation sufficiently depolarizes the postsynaptic membranes, which reduces the extent of the \( \text{Mg}^{2+} \)-induced block of NMDA receptors and allows \( \text{Ca}^{2+} \) influx (15). Consistent with this model, the PSD-95-interacting NMDA receptors also showed a voltage-dependent \( \text{Mg}^{2+} \) block of the channels (data not shown). A number of evidences indicate that the activation of protein kinase C is necessary for induction of LTP (15, 41). If the activation of protein kinase C is induced by

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**FIG. 7.** Expression of mutant PSD-95 in oocytes. A, schematic diagram of the wild-type and mutant constructs of PSD-95 used in this study. The domains of PSD-95 are indicated. S-S, substitution of cysteines 3 and 5 with serine. B and C, immunoblotting of wild-type and mutant PSD-95 expressed in oocytes. Wild-type or mutant PSD-95 cRNA (25 ng) was injected into an oocyte expressing the \( \varepsilon_2/\zeta_1 \) NMDA receptor. Immunoblotting was performed as described under “Experimental Procedures” using polyclonal antibodies against the NH\(_2\)-terminal region (amino acids 4–404) (B) and a monoclonal antibody against the central region (amino acids 353–504) (C) of rat PSD-95.

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**FIG. 8.** Effects of wild-type and mutant PSD-95 on the glutamate sensitivity of \( \varepsilon_2/\zeta_1 \) NMDA receptor channels. Wild-type or mutant PSD-95 cRNA (25 ng) was injected into an oocyte expressing the \( \varepsilon_2/\zeta_1 \) NMDA receptor. A ratio of current responses is presented between 100 and 10 \( \mu \text{M} \) t-glutamate in the presence of 10 \( \mu \text{M} \) glycine. The data shown are the mean ± S.E. of the number of oocytes indicated in parentheses. *, \( p < 0.05 \), and **, \( p < 0.01 \) compared with oocytes co-expressing the NMDA receptor with wild-type PSD-95.
tetanic stimulation, PSD-95 may play a protective role regarding the NMDA receptor channels, preventing further potentiation of the channels and excess Ca\textsuperscript{2+} influx.

In this study, we demonstrated that PSD-95 functionally modulates the channel activity of the e\textsuperscript{2}/z heteromeric NMDA receptor. Recently, a number of proteins were reported to bind to PSD-95. GKAP (guanylate kinase-associated protein) or SA-PAP (SAP90/PSD-95-associated protein) binds to the guanylate kinase-like domain (42–44). Neuriligin (45) and CRIP3 (cysteine-rich interactor of PDZ three) (46) bind to the PDZ2 domain. It remains to be seen whether these PSD-95-binding proteins affect the channel modulation by PSD-95.

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