ORIGINAL ARTICLE

Phosphorylation of Delta2 Glutamate Receptors at Serine 945 is Not Required for Cerebellar Long-term Depression

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Abstract: Long-term depression (LTD) of synaptic transmission at parallel fiber (PF)-Purkinje cell synapses is thought to regulate motor learning and memory formation in the cerebellum. Neuronal activity-evoked protein kinase C (PKC) activation is required for the induction of LTD. In addition, the δ2 glutamate receptor (GluRδ2), which is predominantly expressed at PF-Purkinje cell synapses, is indispensable for the induction of LTD; however, the mechanisms by which GluRδ2 regulates LTD and its relationship with PKC activation remain elusive. Interestingly, GluRδ2 is phosphorylated by PKC on serine 945 (Ser945) near its C-terminus and a postsynaptic protein S-SCAM, which could potentially regulate glutamate receptor trafficking and synaptic plasticity, binds to the extreme C-terminus of GluRδ2 in a phosphorylation-dependent manner on Ser945. Here, using a Sindbis-based virus expression approach, we show that a mutant GluRδ2, in which alanine replaced Ser945 and did not undergo PKC phosphorylation, was normally localized at the postsynaptic sites of PF-Purkinje cell synapses. In addition, like wild-type GluRδ2, the phosphorylation-disrupted GluRδ2 successfully rescued abrogated LTD in *GluRδ2*-null Purkinje cells. These results indicate that Ser945, a major PKC phosphorylation site of of GluRδ2, may not play a crucial role in induction of LTD in the cerebellum. (Keio J Med 57 (2) : 105–110, June 2008)

Key words: cerebellum, Purkinje cell, long-term depression, δ2 glutamate receptor, phosphorylation

Introduction

Information storage in neural circuits depends on neuronal activity-dependent persistent changes in neurotransmission efficiency, such as long-term potentiation and long-term depression (LTD). Experimental studies have established that LTD in the cerebellum is a major mechanism responsible for motor learning.[1] Cerebellar Purkinje cells receive two excitatory inputs from parallel fibers (PFs; axons of granule cells) and climbing fibers (CFs; axons of inferior olivary neurons); according to the LTD theory, PF responses are persistently depressed when they are repeatedly evoked together with performance error-encoding CF activities. Since LTD is observed in various brain regions, an understanding of cerebellar LTD is not only essential for an appreciation of normal and abnormal cerebellar functions, but also provides key insights into basic mechanisms underlying synaptic plasticity.

Like LTD in other brain regions, cerebellar LTD is thought to be caused by the selective endocytosis of the postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA)-subtype of glutamate receptors that normally mediate synaptic transmission.[2,3] Nevertheless, cerebellar LTD is unique in that it absolutely requires the presence of another class of glutamate receptor, the δ2 glutamate receptor (GluRδ2), which is predominantly expressed at PF-Purkinje cell synapses.[4] Indeed, LTD is completely absent in *GluRδ2*-null mice[5,6] and the application of an antibody against GluRδ2's extracellular

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N-terminal region to wild-type Purkinje cells rapidly induced the endocytosis of AMPA receptors and abrogated the further induction of LTD.[7] Interestingly, although GluRδ2 belongs to the ionotropic glutamate receptor family, a GluRδ2 transgene, in which the putative channel pore domain was disrupted, rescued all abnormal phenotypes of *GluRδ2*-null mice.[8,9] In contrast, the extreme C-terminal end of GluRδ2, which binds to various intracellular proteins like PSD-93,[10] PTPMEG,[11] S-SCAM/MAGI-2,[12] and delphilin,[13] was indispensable for the induction of LTD and motor learning. These results indicate that GluRδ2 may function as a nonionotropic receptor of which C-terminus controls the number of postsynaptic AMPA receptors and regulates LTD; however, the precise mechanisms by which GluRδ2 regulates LTD remain elusive.

LTD can be induced by direct protein kinase C (PKC) activation in Purkinje cells.[14,15] In addition, conjunctive stimulation of PFs and CFs could not induce LTD when PKC activities were blocked in Purkinje cells,[16] indicating that PKC activation in Purkinje cells is necessary and sufficient for LTD induction. Indeed, recent imaging studies indicated that PKCα is activated during LTD induction in cultured Purkinje cells.[17] One of the crucial substrates of PKC in Purkinje cells during LTD is the C-terminus of the AMPA receptor subunit GluR2.[2,18] In addition, we previously demonstrated that GluRδ2 was phosphorylated at Ser945 by PKC *in vitro* and by an LTD-inducing stimulus in slice preparations.[19] Interestingly, the binding of a synaptic scaffolding molecule, S-SCAM, to the extreme C-terminus of GluRδ2 was shown to be significantly enhanced by the phosphorylation of Ser945.[12] Since S-SCAM is associated with several synaptic proteins including transmembrane AMPA receptor regulatory proteins (TARPs),[20] which regulate AMPA receptor localization at synapses,[21] we hypothesized that the S-SCAM signaling pathway, which could be controlled by the PKC phosphorylation status of GluRδ2's C-terminus, may be involved in the regulation of LTD induction. To test this hypothesis, we employed a virus-mediated "rescue" approach[22] using a mutant GluRδ2 in which alanine replaced Ser945 (GluRδ2^{S945A}). We demonstrated that like wild-type GluRδ2, GluRδ2^{S945A} was expressed at postsynaptic sites and rescued abrogated LTD in *GluRδ2*-null Purkinje cells. These results indicate that the PKC phosphorylation site Ser945 does not play a major role in the induction of LTD.

Materials and Methods

Vector constructs and virus production

A modified Sindbis virus vector was used, as previously described.[22] The second subgenomic promoter was inserted into pSinRep5 (Invitrogen, Carlsbad, CA) to express a yellow fluorescent protein (YFP; Invitrogen) for the identification of the infected cells.

In vivo microinjection

GluRδ2-null mice were anesthetized using an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (20 mg/kg; Sigma, St. Louis, MO). A small hole was made in the occipital bone with a dental drill, and the dura mater was ablated. A glass pipette (30 μm in diameter) was inserted into the cerebellum 900 μm from the surface, and 250 nL of virus solution was injected using a Nanoliter (World Precision Instruments, Sarasota, FL). The mice were sacrificed 24-48 hours after injection. All the experimental procedures involving the use of animals were approved by the Animal Resource Committee of the School of Medicine, Keio University.

Immunohistochemistry

The immunohistochemical preparations were performed as described previously.[22] Under deep anesthesia with an intraperitoneal injection of pentobarbital, the virus-injected mice were fixed by cardiac perfusion with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS, pH 7.4), and the cerebellum was removed and post-fixed by 4% paraformaldehyde in PBS overnight. Parasagittal slices (150 μm thick) were prepared using a microslicer (Dosaka EM, Kyoto, Japan). Then, the slices were treated with 1 mg/mL of pepsin (DAKO) in 0.2 N HCl at 37°C for 20 min. After being permeabilized by 0.4% Triton X-100 in PBS with 0.2% normal goat serum and 0.2% bovine serum albumin at 4°C overnight, the sections were treated with antibodies against GluRδ2 (kindly donated by Dr. Watanabe, Hokkaido Univ., Japan) and YFP (Invitrogen). For visualization, secondary antibodies conjugated with Alexa 594 and 488 (Molecular Probes, Eugene, OR) were used.

Electrophysiology

Parasagittal cerebellar slices (200 μm thick) were prepared from *GluRδ2*-null mice (postnatal day 17-28) using a linear slicer (Dosaka EM). Whole-cell voltage-clamp recordings from YFP-positive Purkinje cells were performed as described previously.[7] Excitatory postsynaptic currents (EPSCs) at PF-Purkinje cell synapses (PF-EPSCs) were evoked by stimulating the molecular layer (pulse width, 200 μsec) with a glass pipette filled with 140 mM NaCl and 10 mM HEPES. Stimulation of the PFs was confirmed by the paired-pulse facilitation of PF-EPSCs with a 50 msec inter-stimulus interval. Purkinje cells were voltage-clamped at -80 mV, and PF-EPSCs evoked at 0.1 Hz were monitored. After obtaining stable recordings of the PF-EPSCs for at least 10 min, the Pur-

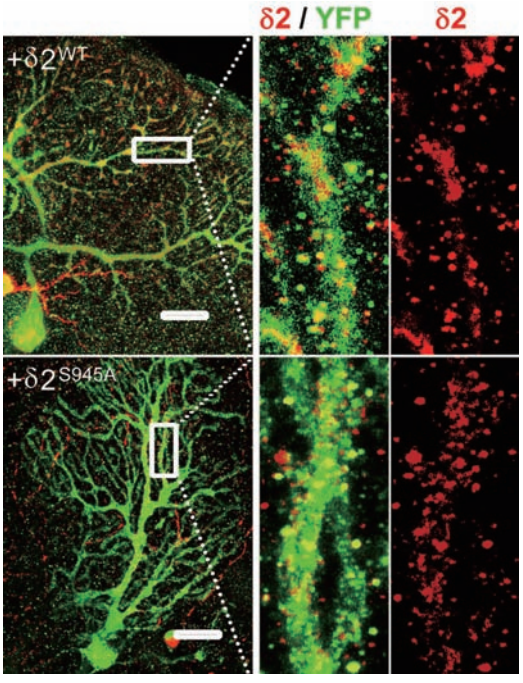

Fig. 1 Distribution patterns of virally transduced wild-type GluRδ2 (GluRδ2^{WT}) and GluRδ2^{S945A} in *GluRδ2*-null cerebellar slices. GluRδ2 immunoreactivities were visualized using Alexa 594 (red); immunoreactivities against YFP, visualized with Alexa 488 (green), were overlaid. Scale bars, 20 µm.

kinje cells were depolarized to 10 mV for 200 msec at 1 Hz 30 times in conjunction with evoking the PF-EPSCs. The measurements were discarded when the series resistance changed by 20% of its original value. The current traces were filtered at 2 kHz and digitized at 10 kHz.

For the statistical analysis, the EPSC amplitudes were averaged every 1 min. These averaged EPSCs were normalized by those for 1 min before the conjunctive stimuli.

Results and Discussion

We employed a Sindbis virus vector to express wild-type GluRδ2 and YFP (Sin-GluRδ2^{wt}-Y), GluRδ2^{S945A} - in which alanine had replaced the PKC phosphorylation site Ser945 - and YFP (Sin-GluRδ2^{S945A}-Y), or YFP alone (Sin-Y). Twenty-four to 48 hours after injection into lobule VI or VII of the cerebellar vermis of *GluRδ2*-null mice, we observed YFP signals and GluRδ2 immunoreactivities mainly in Purkinje cells in acutely prepared cerebellar slices, as previously reported (Fig. 1).[22] Although many neurotransmitter receptors are anchored at postsynaptic membranes via an interaction with scaffolding proteins at their C-termini, we previously demonstrated that a virally expressed mutant GluRδ2 lacking the C-terminal ends normally localized at the postsynaptic membranes.[22] To confirm that GluRδ2^{S945A} was also expressed at postsynaptic sites, we performed an immunohistochemical analysis of *GluRδ2*-null Purkinje cells infected with GluRδ2^{wt} or GluRδ2^{S945A}. Confocal microscopic studies showed no difference in localization patterns within dendrites and spines between GluRδ2^{wt} and GluRδ2^{S945A} (Fig. 1). Therefore, the PKC phosphorylation site of the C-terminus of GluRδ2 is unlikely to be essential for localizing GluRδ2 at synapses.

To examine whether GluRδ2^{S945A}, like GluRδ2^{wt}, could rescue abrogated LTD at PF-Purkinje cell synapses in *GluRδ2*-null cerebellum, we performed whole-cell voltage-clamp recordings in virally transduced acute cerebellar slices. Sindbis virus infection itself did not significantly affect the basic membrane properties of the Purkinje cells or the kinetics of the PF-evoked EPSCs in the Purkinje cells (Table 1). In addition, the probability of presynaptic transmitter release, as assessed by measuring the paired-pulse facilitation of PF-EPSCs,[23] was similar among *GluRδ2*-null Purkinje cells infected with Sin-GluRδ2^{wt}-Y, Sin-GluRδ2^{S945A}-Y, or Sin-Y (Table 1). Finally, we examined whether LTD could be restored in *GluRδ2*-null Purkinje cells infected with Sin-GluRδ2^{S945A}-Y. To exclude any effect from abnormal CF innervation in *GluRδ2*-null Purkinje cells, we replaced CF stimulation with direct depolarization of the Purkinje cells.[24] In *GluRδ2*-null Purkinje cells infected with Sin-GluRδ2^{wt}-Y (Fig. 2A) or Sin-GluRδ2^{S945A}-Y (Fig. 2B), this protocol successfully induced LTD in PF-EPSCs, but it did not induce LTD in Purkinje cells expressing YFP (Fig. 2C). The amplitude of PF-EPSCs 26 - 30 min after conjunctive stimuli was 64 ± 4% (n = 5 from 3 mice) of the control responses in Purkinje cells infected with Sin-GluRδ2^{wt}-Y and 66 ± 8% (n = 5 from 4 mice) in cells infected with Sin-GluRδ2^{S945A}-Y, whereas that of cells treated with Sin-Y was 96 ± 8% (n = 5 from 4 mice; Student *t*-test, $p < 0.001$ vs. Sin-GluRδ2^{wt}-Y or Sin-GluRδ2^{S945A}-Y; Fig. 2D). In contrast, we previously showed that Sindbis virus-based expression of GluRδ2 that had a mutation in the channel pore domain (GluRδ2-VR) or GluRδ2 that lacked the C-terminal seven amino acids (GluRδ2-ΔCT7) could not rescue the LTD phenotype in *GluRδ2*-null Purkinje cells in acute slice preparations,[8,9] indicating that LTD could not be rescued by overexpression of mutant GluRδ2 proteins. These results suggest that PKC phosphorylation site Ser945 is not essential for GluRδ2 to regulate LTD induction in acute slice preparations.

Although GluRδ2 is crucial for LTD and motor learning in the cerebellum, the mechanisms by which GluRδ2 participates in cerebellar functions have been elusive.[4] We previously demonstrated that abrogated LTD was restored in *GluRδ2*-null Purkinje cells by introducing wild-type GluRδ2, but not a mutant GluRδ2-ΔCT7, to which PSD-93, PTP-MEG, delphilin and S-SCAM cannot bind.[22,25]

Table 1 Electrophysiological properties of virus-infected Purkinje cells*

Construct / Background (number)	**Sin-δ2^{Wt}-Y** / *GluRδ2*-null (n = 13)	**Sin-δ2^{S945A}-Y** / *GluRδ2*-null (n = 11)	**Sin-Y** / *GluRδ2*-null (n = 12)
Membrane resistance (MΩ)	80 ± 5	88 ± 5	77 ± 6
Membrane capacitance (pF)	594 ± 43	581 ± 32	596 ± 48
PF-EPSC Rise time** (ms)	3.6 ± 0.2	3.2 ± 0.2	3.4 ± 0.4
PF-EPSC Decay time*** (ms)	22 ± 2	25 ± 2	22 ± 2
PF-EPSC PPF ratio****	2.2 ± 0.2	2.1 ± 0.1	1.9 ± 0.1

*Data represent mean ± SEM. There were no statistically significant differences among groups.
**The time required for the synaptic current to increase from 10% to 90%.
***Current decay was fitted to single exponential curves.
****Paired-pulse facilitation (PPF) ratio of PF-EPSCs was defined as the ratio of the amplitude of the second EPSC to that of the first EPSC, when the two stimuli were given at 50 ms intervals.

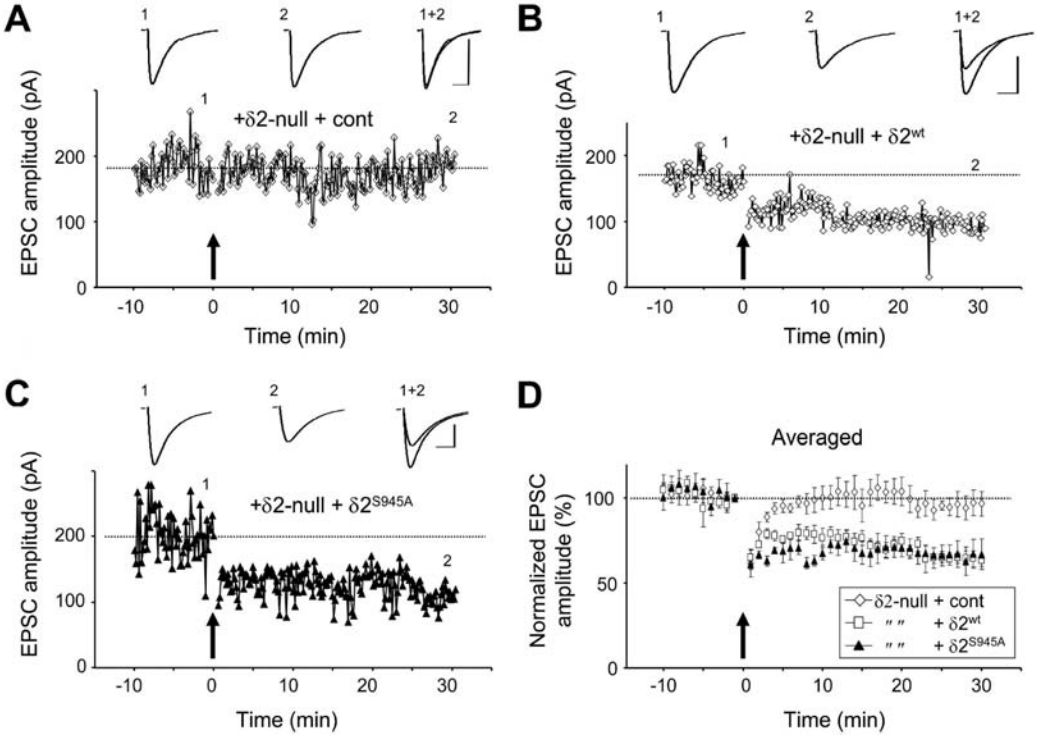

Fig. 2 Virally introduced GluRδ2^{S945A} rescued LTD induction in *GluRδ2*-null Purkinje cells. (**A-C**) LTD induction in *GluRδ2*-null Purkinje cells infected with various vectors. LTD could not be induced in *GluRδ2*-null Purkinje cells by the expression of YFP (cont; A), but it could be induced by the expression of wild-type GluRδ2 (δ2^{wt}; **B**) and a mutant GluRδ2 in which Ala replaced Ser 945 (δ2^{S945A}; **C**). LTD-inducing conjunctive stimuli were applied at the time point indicated by the vertical arrows. The sweeps in the inset indicate the EPSCs at t = 0 (1), t = 30 min (2), and the merged traces (1+2). Scale bars: 100 pA, 25 ms. (**D**) Averaged EPSCs in *GluRδ2*-null Purkinje cells infected with various vectors. EPSCs were normalized by the averaged EPSCs from -1 to 0 min prior to the conjunctive stimuli. Each point represents the mean and standard errors of normalized EPSC (*GluRδ2*-null Purkinje cells expressing control YFP (◊, n = 5), δ2^{wt} (□, n = 5) and GluRδ2^{S945A} (▲, n=5). The LTD-inducing conjunctive stimulus was applied at the time point indicated by a vertical arrow.

We also previously indicated that PSD-93 and delphilin were dispensable for LTD induction.[22] In this study, we demonstrated that GluRδ2^{S945A}, which could not be phosphorylated by PKC[19] and could not strongly interact with S-SCAM,[12] successfully rescued abrogated LTD at PF-Purkinje cell synapses in *GluRδ2*-null cerebellum. Therefore, although we cannot completely rule out the possibility that overexpression of a mutant GluRδ2^{S945A} protein may have compensated its reduced binding to S-SCAM, GluRδ2-S-SCAM interaction is unlikely to play a major role in LTD induction. Instead, we propose that other molecules that bind to the C-terminal end of GluRδ2, such as PTPMEG, may be crucial for the induction of LTD.

Even if the GluRδ2-S-SCAM interaction may not play a major role in LTD induction, it may serve for other functions of GluRδ2. For example, presynaptic elements are often misaligned with, or detached from, their opposing postsynaptic counterparts at PF-Purkinje cell synapses in *GluRδ2*-null cerebella.[26] As S-SCAM assembles with the adhesion molecule neuroligin-1, which induces presynaptic differentiation by binding to a presynaptic protein neurexin,[27] GluRδ2 may regulate the adhesion of pre- and post-synaptic elements at PF-Purkinje cell synapses via S-SCAM. Because of the cytotoxicity of the Sindbis virus, we could not observe the longer-term effects of GluRδ2 transduction on these morphological phenotypes.[22] Thus, further studies utilizing other virus vectors with attenuated toxicity or a transgenic mice-based rescue approach will be required to elucidate the longer-term functions of GluRδ2-S-SCAM interaction and its possible regulation by PKC.

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