Phospholipase Cβ4 and Protein Kinase Cα and/or Protein Kinase CβI Are Involved in the Induction of Long Term Depression in Cerebellar Purkinje Cells*

Moritoshi Hirono‡‡, Takashi Sugiyama‡, Yasushi Kishimoto‡, Ikuro Sakai**, Takahito Miyazawa‡, Masahiro Kishioti‡, Hiroko Inoue‡, Kazuki Nakaо‡, Masayuki Ikeda‡, Shigenori Kawahara‡, Yutaka Kirino‡, Motoya Katsuki§§, Hidenori Horie***, Yoshio Ishikawa**, and Tohru Yoshioka†††† From the ‡Department of Molecular Neurobiology, Advanced Research Institute for Science and Engineering, Waseda University, 3-1-4 Okubo, Shinjuku-ku, Tokyo 169-8555, the §§Department of Molecular Neurobiology, School of Human Sciences, Waseda University, 2-7-5-60 Mitajima, Tokorozawa-shi, Saitama 359-1192, the |||Laboratory of Neurophysiology, School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, the §§§Department of Physiology, School of Medicine, Yokohama City University, 3-9 Puku-ura, Kanazawa-ku, Yokohama 236-0004, the ‡‡‡Department of Neurosurgery, National Defense Medical College, 3-2 Namiki, Tokorozawa-shi, Saitama 359-8513, the ‡‡‡‡Department of DNA Biology and Embryo Engineering, Research Center of Animal Models for Human Diseases, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

Activation of the type-1 metabotropic glutamate receptor (mGluR1) signaling pathway in the cerebellum involves activation of phospholipase C (PLC) and protein kinase C (PKC) for the induction of cerebellar long term depression (LTD). The PLC and PKC isoforms that are involved in LTD remain unclear, however. One previous study found no change in LTD in PKCγ-deficient mice, thus, in the present study, we examined cerebellar LTD in PLCβ4-deficient mice. Immunohistochemical and Western blot analyses of cerebellum from wild-type mice revealed that PLCβ1 was expressed weakly and uniformly, PLCβ2 was not detected, PLCβ3 was expressed predominantly in caudal cerebellum (lobes 7–10), and PLCβ4 was expressed uniformly throughout. In PLCβ4-deficient mice, expression of total PLCβ, the mGluR1-mediated Ca2+ response, and LTD induction were greatly reduced in rostral cerebellum (lobes 1–6). Furthermore, we used immunohistochemistry to localize PKCα, βI, and γ in mouse cerebellar Purkinje cells during LTD induction. Both PKCα and PKCβI were found to be translocated to the plasmamembrane under these conditions. Taken together, these results suggest that mGluR1-mediated activation of PLCβ4 in rostral cerebellar Purkinje cells induced LTD via PKCα and/or PKCβI.

Cerebellar long term depression (LTD)1 is produced by associative activation of parallel fiber (PF) and climbing fiber synapses (1–4), which results in co-activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) and type-1 metabotropic glutamate receptors (mGluR1) in Purkinje cells followed by activation of phospholipase C (PLC) coupled to Gq, hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol, an increase in the concentration of intracellular Ca2+ ([Ca2+]i), and activation of protein kinase C (PKC; for review, see Ref. 5).

As predicted, mGluR1-deficient mutant mice exhibit impaired cerebellar LTD (6, 7), however, there is no disruption of LTD in PKCγ-deficient mice (8). These results raise the possibility that disruption of one of the intermediate molecules in the mGluR1 signaling pathway may disrupt LTD. Of the four isoforms of PLC, PLCβ1–4 (9, 10), two are abundant in the cerebellum, PLCβ3 and PLCβ4 (11–13). PLCβ3 is expressed predominantly in the caudal half of cerebellar Purkinje cells, whereas PLCβ4 is distributed throughout cerebellar Purkinje cells. Fly homologue of PLCβ4 has been implicated in transduction of visual information in Drosophila photoreceptors (14, 15), however, the role of PLCβ4 in the cerebellum remains unknown. The PLCβ4-deficient mice were viable but had a higher mortality rate than wild-type mice, and the body weight of PLCβ4-deficient mice was generally less than that of wild-type mice in the early stages of postnatal development, as reported previously (16, 17). The body weight of the PLCβ4-deficient mice gradually increased to match wild-type mice 8 weeks after birth. Using a light microscope, no differences were detected in the size of whole cerebellum, lobe size, or Purkinje cell size between PLCβ4-deficient mice and wild-type mice. The body weight of the PLCβ4-deficient mice gradually increased to match wild-type mice 8 weeks after birth. Using a light microscope, no differences were detected in the size of whole cerebellum, lobe size, or Purkinje cell size between PLCβ4-deficient and wild-type mice. Anatomical alterations are minimal in mGluR1-deficient mutant mice (6) and cerebellar architecture is also normal in glial fibrillary acidic protein (GFAP)-deficient mutant mice (18). Only one abnormality in the cerebellar anatomy of PLCβ4-deficient mice...
has been reported so far; persistent multiple climbing fiber innervation of Purkinje cells (19), which has also been reported in mGlur1−/−, GluR2−/−, and PKCγ-deficient but not GFAP-deficient mice (18, 20–22). Eight PKC isozymes (α, β, δ, ε, ζ, and κ) are expressed in the cerebellum, of which six (α, β, γ, δ, ε, and ζ) are found in cerebellar Purkinje cells (23–25). Selective expression of a pseudosubstrate PKC inhibitor, PKC inhibitor peptide (Arg19-Val31), in Purkinje cells completely blocked cerebellar LTD (26). Therefore, using PLCβ4-deficient mice in the present study, we examined the effects of disruption of PLCβ4 on cerebellar LTD and determined which PKC isozymes were essential for the induction of LTD.

EXPERIMENTAL PROCEDURES

Generation of PLCβ4-deficient Mice—Mice with a disruption of the PLCβ4 gene were generated in the laboratory of M. Katsuki according to standard methods (27). A genomic clone encoding the PLCβ4 catalytic region (denoted the Y region) was isolated to construct a targeting vector in which exons that encode amino acid residues 539–646 were replaced by transmembrane and cytoplasmic domains of the human major histocompatibility complex Class II beta chain. A 1.4-kb fragment of the mouse Y region was ligated into the Y region of the targeting vector. The resulting construct was electroporated into 129SvJ embryonic stem (ES) cells. Embryonic stem cells were transfected with the targeting vector by electroporation and selected with ganciclovir (250 μg/ml) for 8 days. G418-resistant colonies were isolated, and the targeted clones were selected using genomic Southern blot analysis with a probe as illustrated in Fig. 1A. Chimeric mice were generated from frozen C57BL/6J blastocysts injected with the embryonic stem cells after warming (28). Male chimeric mice were mated with C57BL/6J female mice. The tail DNA of offspring was analyzed using Southern blot analysis (Fig. 1B) to identify the genotype or amplified using polymerase chain reaction.

Phospholipase C Assay—PLC enzymatic activity was quantified in 200 μl of assay mixture containing 150 μM PIP2. The mixture contained 20,000 cpm [3H]PIP2, 1 mM EGTA, 10 mM CaCl2, 0.1% sodium deoxycholate, 1 mg/ml bovine serum albumin, and 50 mM HEPES, pH 6.8. The reaction mixture was incubated at 37 °C and centrifuged (10,000 × g for 30 min) to precipitate the cerebellar homogenate, and the reaction was terminated as previously described (29).

Western Blot Analysis and Immunohistochemistry—Each lobe of the vermis of the cerebellum from wild-type and PLCβ4-deficient mice was homogenized, and 3 μg of protein was separated using 7.5% SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to a nitrocellulose membrane. The membrane was incubated with anti-PLCγ, α-β, δ, ε, and ζ antibodies (1/1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) and then with an alkaline-phosphatase-conjugated goat antibody (1/2000 dilution, Promega, Madison, WI). Immunoreacted bands were visualized using Protoblot Western Chemiluminescent substrate kit (Amersham Pharmacia Biotech, Piscataway, NJ) and exposed to X-ray film. The membranes were then exposed to X-ray film for 1 h with 0.001% Cremophore EL. The slices were then maintained in ACSF for at least 30 min and transferred to the stage of an Axionlab 2 microscope (Zeiss, Germany). Fluorescence Ca2+ ratio imaging was carried out by excitation of the indicator at 340–380 nm, and paired emission images were acquired using a cooled charged-coupled device camera (C4880, Hamamatsu Photonics, Japan) at 510 nm. Fluorescence images were acquired using a 60× water immersion objective (UPLANAPF, numerical aperture (NA) 0.90, Olympus, Japan) that efficiently passed 340–380 nm light, and ratios were determined using a digital image acquisition system and image-processing software (ARGUS 50/CA, Hamamatsu Photonics, Japan).

Electrophysiology—Whole-cell voltage-clamp recordings were made from visually identified Purkinje cells under Nomarski optics using a 40× water immersion objective (NA 0.75, Zeiss). Patch pipettes (3–4 MΩ) were filled with intracellular solution containing 150 mM KCNSO4, 5 mM KCl, 0.3 mM K-EGTA, 5.0 mM sodium HEPES, 3.0 mM MgATP, and 0.4 mM NaN-GTP (pH 7.4). Membrane currents were recorded using an EPC-7 amplifier (List Electronics, Darmstadt, Germany) and a CLAMP software (Axon Instruments, Union City, CA), digitized, and stored on a computer disc for off-line analysis. PF-mediated ionotropic-glutamate-receptor-type excitatory postsynaptic currents (EPSCs) were identified based on response properties following paired-pulse stimulation (duration, 50–100 μs; amplitude, 5–15 V) applied via a glass microelectrode with 2- to 3-μm tip diameter, filled with normal ACSF, and placed within the molecular layer in the cerebellar cortex. Paired-pulse stimulation was applied at 0.2 Hz. For measuring PF-evoked EPSCs, bicculline (10 μM) was added to the ACSF to eliminate γ-aminobutyric acid (GABA)-mediated postsynaptic currents. Series resistance (8–15 MΩ) was monitored using a −5-mV hyperpolarizing voltage step after PF stimulation. The series resistance compensation control of the amplitude was set between 60 and 70%. Membrane-compensated EPSCs were obtained at PF frequency stimulation (10 pulses at 100 Hz, duration, 180 μs; amplitude, 30 V). To prevent ionotropic glutamate and GABAγ receptor responses, 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM), (−)-2-amino-5-phosphonopentanoic acid (30 μM), and bicculline (50 μM) were added at the external solution. All physiological experiments were performed at room temperature.

Immunohistochemical Staining—The experimental protocols for LTD were performed as described previously with slight modification (32, 33). Briefly, sagittal slices (400 μm thick) of cerebellar vermis were prepared from 3- to 5-week-old wild-type and PLCβ4-deficient mice using a microslicer (DTK-1000, Dosaka, Japan) and maintained at room temperature in artificial cerebrospinal fluid (ACSF), which consisted of 138.6 mM NaCl, 3.35 mM KCl, 21 mM NaHCO3, 0.6 mM NaHPO4, 9.9 mM glucose, 2.5 mM CaCl2, and 1 mM MgCl2 and was gassed with a mixture of 95% O2 and 5% CO2 (pH 7.4). The Ca2+ indicator fura-2 (1 mm, Dojin, Japan) was injected into Purkinje cells for 2–3 min through patch pipettes, and biellamellar BODIPY was added in 10 μM fura-2 AM (Dojin) for 1 h with 0.001% Cresylmethyl E. The slices were then maintained in ACSF for at least 30 min and transferred to the stage of an Axioplan 2 microscope (Zeiss, Germany). Fluorescence Ca2+ ratio imaging was carried out by excitation of the indicator at 340–380 nm, and paired emission images were acquired using a cooled charged-coupled device camera (C4880, Hamamatsu Photonics, Japan) at 510 nm. Fluorescence images were acquired using a 60× water immersion objective (UPLANAPF, numerical aperture (NA) 0.90, Olympus, Japan) that efficiently passed 340–380 nm light, and ratios were determined using a digital image acquisition system and image-processing software (ARGUS 50/CA, Hamamatsu Photonics, Japan).

Biochemical and Histological Characterization of Cerebellar PLCγ—The total activity of membrane-associated PLC was examined using [3H]PIP2 as a substrate in cerebellar slices from wild-type (n = 4) and PLCβ4-deficient mice (n = 5). As shown in Fig. 1C, the total PLC activity in PLCβ4-deficient mice was less than 30% of control values in rostral cerebellum and less than 40% in caudal cerebellum. These data suggest that PLCβ4 activity in rostral and caudal cerebellum was 70 and 60% of the total PLC activity, respectively. Total PLC activity was found to be 5.6 nmol/min/mg in rostral cerebellum and 4.5 nmol/min/mg in caudal cerebellum.
Western blot analysis (Fig. 2A) indicates that PLCβ1, PLCβ3, and PLCβ4 were expressed in wild-type mouse cerebellum. PLCβ4 protein was not detected in the cerebellum from PLCβ4-deficient mice (Fig. 2A), whereas the expression levels of the other PLCβ isoforms (PLCβ1, PLCβ2, and PLCβ3) were not altered.

Immunohistochemical analysis was performed using an anti-PLCβ4 antibody (Fig. 2, B and C). Each of the lobes in the cerebellar slices was numbered from 1 to 10 as shown in Fig. 2C. PLCβ4 was expressed uniformly in Purkinje cells in rostral (lobes 1–6) and caudal (lobes 7–10) cerebellum from wild-type mice (Fig. 2C), whereas PLCβ3 is more abundant in Purkinje cells in caudal cerebellum from wild-type mice (13, 19; Fig. 2D–F). No morphological changes were observed in the cerebellum of PLCβ4-deficient mice when examined using light microscopy (data not shown).

Normal PF-Purkinje Cell Synaptic Transmission in PLCβ4-deficient Mouse Cerebellum—To examine PF-Purkinje cell synaptic function in PLCβ4-deficient mice, we measured the rise and decay time constants of EPSCs, which were calculated using a single-exponential fit (34) and paired-pulse facilitation in acute cerebellar slices. The mean rise time constant was 1.23 ± 0.06 ms (n = 30) and 1.21 ± 0.05 ms (n = 35) in Purkinje cells from wild-type and PLCβ4-deficient mice, respectively. The mean decay time constant was 14.1 ± 0.5 ms (n = 30) in wild-type versus 12.8 ± 0.5 ms (n = 35) in PLCβ4-deficient Purkinje cells. There was no significant difference in either the rise or decay time constants between wild-type and PLCβ4-deficient mice (p > 0.05; Fig. 3, A and B). The PF responses exhibited paired-pulse facilitation (35), which decreased with increasing interpulse intervals in a similar manner in wild-type and PLCβ4-deficient mice (Fig. 3C). Therefore, short term plasticity in PF-Purkinje cell synapses appeared normal in PLCβ4-deficient mice. Furthermore, no significant difference was found in the resting membrane potentials (−55.5 ± 1.3 mV versus −56.3 ± 1.5 mV) of Purkinje cells from wild-type and PLCβ4-deficient mice.

LTD Was Not Inducible in Rostral Cerebellum from PLCβ4-deficient Mice—LTD of synaptic transmission at PF-Purkinje cell synapses is induced by simultaneous low frequency activation of PF and climbing fibers (1, 3). Climbing fiber stimulation
from 10 mice) was comparable to LTD in wild-type mice (Mann-Whitney test, *p* < 0.05) in rostral cerebellum from PLCβ4-deficient mice, an area in which PLCβ1 and PLCβ3 were also not expressed strongly in these mutant mice. Unfortunately, we could not determine the coupling selectivity between PLC (β3 and β4) and PKC (α and β1), because imaging of PKC in caudal part is not clear (data not shown). To overcome this difficulty, a real time imaging of GFP-labeled PKC in living cells under LTD condition is desirable, but it is impossible at present stage. Therefore, we concluded that, at the lowest estimate, both PKCα and PKCβII were translocated during LTD induction, but PKCγ was not.

**DISCUSSION**

In the present study, the mGluR1-mediated Ca2+ response and LTD induction was greatly reduced in the rostral cerebellum from PLCβ4-deficient mice, an area in which PLCβ1 and PLCβ3 were also not expressed strongly in these mutant mice. In the caudal cerebellum, however, the residual PLCβ3 activity was sufficient to generate Ca2+ elevation and LTD induction. These results suggest that there was a minimum level of PLCβ3 and PLCβ4 required to generate the mGluR1-mediated Ca2+ response and LTD. We also showed that LTD induction in rostral and caudal cerebellum required activation of classic PKC isoforms.

**Fig. 3.** The mean rise and decay times and paired-pulse facilitation of Purkinje cell synaptic responses were not affected in PLCβ4-deficient mice. *A*—*C*, PF-EPSCs were unaltered in Purkinje cells from PLCβ4-deficient mice. Representative traces showing the response to paired-pulse stimulation in a Purkinje cell from a wild-type mouse (*A*) and a PLCβ4-deficient mouse (*B*). Each trace is the average of 12 consecutive EPSCs. The holding potential was −60 mV. *C*, paired-pulse facilitation of PF-EPSCs (expressed as the ratio of the responses to the first and second pulses) in Purkinje cells from wild-type (open circle; *n* = 10, from six mice) and PLCβ4-deficient (solid circle; *n* = 12, from six mice) mice is plotted as a function of interpulse interval. Data points represent the mean ± S.E.

PKC isozymes in Purkinje cells from PLCβ4-deficient mice were examined as a function of mGluR1-mediated IP3-dependent Ca2+ mobilization. Only classic PKC isozymes (α, β1, βII, and γ) can be activated by IP3-activated Ca2+ release and diacylglycerol (38). Although application of the mGluR1-specific agonist (RS)-3,5-dihydroxyphenylglycine (DHPG) has been shown to increase [Ca2+]i, in rodent cerebellar Purkinje cells (39; Fig. 5K), in the present study in PLCβ4-deficient mice, DHPG did not induce Ca2+ mobilization (*n* = 3; Fig. 5, C and E) in lobe 6 Purkinje cells but increased dendritic [Ca2+]i to a small degree in lobe 9 Purkinje cells (*n* = 4; Fig. 5, H and J).

To exclude the possibility that the lack of Ca2+ release in the mutant mice was an artifact of slice preparation, we examined AMPAR-induced Ca2+ release after DHPG stimulation. Application of AMPA evoked a large Ca2+ transient in Purkinje cells in wild-type cerebellum (Fig. 5K). As shown in Fig. 5 (E and J), large Ca2+ responses were also obtained in Purkinje cells in rostral and caudal cerebellum from PLCβ4-deficient mice following application of AMPA. There was an additional slow phase of the AMPA-induced Ca2+ response in the dendrite (Fig. 5, E, J, and K), which may be due to Ca2+ signals traveling from distal parts of the dendrite. In the soma, however, the two phases overlapped. These results suggest that classic PKC isoforms were not activated in rostral cerebellum from PLCβ4-deficient mice.

To investigate possible colocalization of classic PKC isoforms with PLCβ4, we examined the distribution of classic PKC isoforms using antibodies against each isozyme. Immunostaining with antibodies were done as described under “Experimental Procedure.” As shown in Fig. 6 (A–D), PKCα, βI, and γ were expressed uniformly in Purkinje cells, whereas PKCβII was not detected. These data are consistent with data obtained previously by several authors (23, 25). To investigate the PKC isoforms coupled to PLCβ4 and PLCβ3, we examined the translocation of PKC isoforms during LTD induction using immunohistochemistry. Fluorescence-labeled secondary antibodies were used in this experiment, because fluorescent images showed a relatively large difference between wild-type and PLCβ4-deficient mice with high contrast. 400-μm cerebellar slices from wild-type (*n* = 8 from four mice) and PLCβ4-deficient mice (*n* = 8 from four mice) were incubated for 5 min in ACSF with (*n* = 4 of each mice) or without (*n* = 4 of each mice) 100 μM glutamate and 50 mM KCl. After stimulation, samples were rinsed for 5 min, followed by fixation. From 10 to 15 sections (5-μm thickness) from each slice were stained with antibodies. In wild-type mice, there was strong staining for PKCα in the dendrites of Purkinje cells (Fig. 6F), indicating that PKCα is translocated. In contrast, no stain was detected in dendrites in PLCβ4-deficient mice (Fig. 6G). PKCβI immunoreactivity was very strong in Purkinje cell dendrites and soma in all lobes of wild-type mice (Fig. 6I), whereas the fluorescent signal was observed only in cell somas in rostral part of PLCβ4-deficient mice (Fig. 6J). No difference in staining for PKCγ, however, was detectable between wild-type and PLCβ4-deficient mice (Fig. 6, L and M).

Unfortunately, we could not determine the coupling selectivity between PLC (β3 and β4) and PKC (α and β1), because imaging of PKC in caudal part is not clear (data not shown). To overcome this difficulty, a real time imaging of GFP-labeled PKC in living cells under LTD condition is desirable, but it is impossible at present stage. Therefore, we concluded that, at the lowest estimate, both PKCα and PKCβII were translocated during LTD induction, but PKCγ was not.
Differential Functional Localization of PLCβ Isoforms and Intracellular Ca\(^{2+}\) Elevation—We used immunohistochemical and Western blot analyses to localize the PLCβ isoforms in the wild-type mouse cerebellum: PLCβ1 was expressed uniformly and weakly, PLCβ2 was not detected, PLCβ3 was expressed predominantly in caudal cerebellar Purkinje cells (lobes 7–10), and PLCβ4 was expressed uniformly and strongly throughout cerebellar Purkinje cells. These results are consistent with...
previous reports of expression of the corresponding PLCβ isoform mRNA (11-13). In PLCβ4-deficient mice, although PLCβ1 was expressed in rostral cerebellar Purkinje cells, Purkinje cells in rostral cerebellum from PLCβ4-deficient mice lacked the mGluR1-mediated Ca²⁺ response. These results indicate that (i) PLCβ1 is not involved in the mGluR1-mediated signaling pathway in cerebellar Purkinje cells and does not have a role in the induction of cerebellar LTD and (ii) mGluR1-mediated responses in caudal cerebellar Purkinje cells from PLCβ4-deficient mice were produced by activation of PLCβ3 alone. These results suggest that PLCβ4 is a link between the activation of mGluR1 and the induction of LTD in rostral cerebellar Purkinje cells.

**Involvement of PKC Isozymes in the Formation of LTD**—The results of the present study showing that LTD induction was greatly reduced in PLCβ4-deficient mice is consistent with the lack of LTD in cerebellum from mGluR1-deficient mice (6, 7) but does not appear to be consistent with the intact LTD induction observed in PKCγ-deficient mice (8) if PLCβ4 activates PKCγ. Recent evidence using the expression of a PKC inhibitor in Purkinje cells indicates that PKCγ is required for LTD induction (26). PKCδ, ε, and ζ were also expressed in cerebellar Purkinje cells (23); however, these isozymes are Ca²⁺-independent (for review, see Ref. 38), thus, the contribution of these isozymes to LTD induction is likely to be small. Therefore, the remaining isozymes, PKCα and/or PKCβI, may compensate for the lack of PKCγ in rostral cerebellum of PLCβ4-deficient mice.

In PLCβ4-deficient mice, there did not appear to be any compensation for the lack of PLCβ4 by PLCβ1 in the rostral cerebellum. Thus, evidence suggests that, although compensation for deletion of protein isoforms in the signaling pathway downstream of PLCβ occurs, there is no compensatory mechanism for the deletion of PLCβ4 itself.

**Select PKC Translocation during LTD Induction**—Translocation of PKC isoforms after 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulation has been clearly observed in several cell systems (40-44) but not with stimulation sufficient for LTD induction. As described above, the combination of PLC (β3 and β4) activation and translocation of PKC (α and βI) is very likely. Translocation of PKCγ has been observed after stimulation used to induce long term potentiation in neurons in the CA1 region of hippocampus (45, 46) and TPA stimulation in COS-7 cells (43) but not by LTD-forming conditions in Purkinje cells in the present study. This is consistent with previous results from PKCγ-deficient mice (8). This result further indi-
with climbing fibers (19). This difference may underlie the lack of cerebellar LTD induction and eye blink conditioning. PLC (8) report that, in PKC in cerebellum. These ideas are expressed in Fig. 7 as a molecular linkage of combinations between signaling molecules, such as mGluR1-Gq-PLC

It has been reported that Purkinje cells in rostral cerebellum from PLC4-deficient mice form persistent multiple synapses with climbing fibers (19). This difference may underlie the lack of LTD induction in PLC4-deficient mice, however, Chen et al. (8) report that, in PKCγ-deficient mice also, each climbing fiber forms multiple synapses with Purkinje cells and generates multiple spikes that resemble complex spikes, and these mice do exhibit LTD. Thus, the persistent multiple innervation of Purkinje cells by climbing fibers in rostral cerebellum of PLC4-deficient mice does not appear to be involved in LTD induction. Moreover, eye blink conditioning is impaired in PLC4-deficient mice (47). The results from the present study support the idea that induction of LTD has a role in eye blink conditioning, but the developmental shift from multiple to mono-innervation of Purkinje cells by climbing fibers does not have a role in either LTD induction or eye blink conditioning. These ideas are expressed in Fig. 7 as a molecular linkage of mGluR1-Gq-PLC4-PKCa and/or PKCβI.

Taken together, the results obtained in the present study provide strong support for the idea that cerebellar LTD involves PKC activation. Further studies are needed to determine if the signaling pathway involves more specific combinations between signaling molecules, such as mGluR1-Gq-PLC4-PKCa or mGluR1-Gq-PLCβ3-PKCβI.

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Moritsoshi Hirono, Takashi Sugiyama, Yasushi Kishimoto, Ikuo Sakai, Takahito Miyazawa, Masahiro Kishio, Hiroko Inoue, Kazuki Nakao, Masayuki Ikeda, Shigenori Kawahara, Yutaka Kirino, Motoya Katsuki, Hidenori Horie, Yoshihiro Ishikawa and Tohru Yoshioka

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