Characterization of AMPA Receptors Targeted by the Climbing Fiber Transmitter Mediating Presynaptic Inhibition of GABAergic Transmission at Cerebellar Interneuron-Purkinje Cell Synapses

Shin'Ichiro Satake,^{1,2} Si-Young Song,² Qiong Cao,³ Hiromasa Satoh,⁴ Dmitri A. Rusakov,^{2,5} Yuchio Yanagawa,⁶ Eng-Ang Ling,³ Keiji Imoto,¹ and Shiro Konishi⁴

¹National Institute for Physiological Sciences (NIPS), Okazaki 444-8787, Japan, ²Mitsubishi Kagaku Institute of Life Sciences, Tokyo 194-8511, Japan,

³National University of Singapore, 117597 Singapore, ⁴Waseda-Olympus Bioscience Research Institute, 138667 Singapore, ⁵Institute of Neurology, University College London, London WC1N 3BG, United Kingdom, and ⁶Gunma University Graduate School of Medicine, Maebashi 371-8511, Japan

The climbing fiber (CF) neurotransmitter not only excites the postsynaptic Purkinje cell (PC) but also suppresses GABA release from inhibitory interneurons converging onto the same PC depending on AMPA-type glutamate receptor (AMPA) activation. Although the CF/AMPA-mediated inhibition of GABA release provides a likely mechanism boosting the CF input-derived excitation, how the CF transmitter reaches target AMPARs to elicit this action remains unknown. Here, we report that the CF transmitter diffused from its release sites directly targets GluR2/GluR3 AMPARs on interneuron terminals to inhibit GABA release. A weak GluR3-AMPA agonist, bromohomoibotenic acid, produced excitatory currents in the postsynaptic PCs without presynaptic inhibitory effect on GABAergic transmission. Conversely, a specific inhibitor of the GluR2-lacking/ Ca^{2+} -permeable AMPARs, philanthotoxin-433, did not affect the CF-induced inhibition but suppressed AMPAR-mediated currents in Bergmann glia. A low-affinity GluR antagonist, γ -D-glutamylglycine, or retardation of neurotransmitter diffusion by dextran reduced the inhibitory action of CF-stimulation, whereas blockade of glutamate transporters enhanced the CF-induced inhibition. The results suggest that the CF transmitter released after repeated stimulation overwhelms local glutamate uptake and thereby diffuses from the release site to reach GluR2/GluR3 AMPARs on nearby interneuron terminals. Double immunostaining showed that GluR2/3 subunits and glutamate decarboxylase or synaptophysin are colocalized at the perisomatic GABAergic processes surrounding PCs. Finally, electron microscopy detected specific immunoreactivity for GluR2/3 at the presynaptic terminals of symmetric axosomatic synapses on the PC. These findings demonstrate that the CF transmitter directly inhibits GABA release from interneurons to the PC, relying on extrasynaptic diffusion and local heterogeneity in AMPAR subunit compositions.

Key words: AMPA-type glutamate receptor; GABA; climbing fiber; basket cell; Bergmann glia; Purkinje cell; presynaptic inhibition; glutamate transporters; cerebellum

Introduction

GABAergic inhibitory synapses in the brain are subject to profound control by presynaptic regulatory mechanisms after activation of glutamate receptors (GluRs) that include AMPA-, NMDA-, and kainate-type GluRs (Rodríguez-Moreno et al., 1997; Min et al., 1999; Satake et al., 2000; Cossart et al., 2001;

Duguid and Smart, 2004; Engelman and MacDermott, 2004; Huang and Bordey, 2004). A clear physiological role assigned for the GluR-dependent presynaptic modulation is a transient form of synaptic plasticity contributing to modification of synaptic communication. Although substantial evidence has been accumulated regarding the roles of kainate receptors at hippocampal synapses (Kullmann, 2001; Rozas et al., 2003), precise mechanisms by which activation of other GluRs elicits presynaptic regulation of neurotransmission at a number of synapses are much less well understood. For example, it remains unclear whether glutamate released after synaptic activation directly acts on presynaptic GluRs or recruits retrograde signaling through liberation of diffusible messengers from postsynaptic neurons (Alger, 2002; Diana and Marty, 2004; Brenowitz and Regehr, 2005) and/or the neuron-glia signaling pathways (Zhang et al., 2003) to suppress neurotransmitter release.

In the rat cerebellum, the climbing fiber (CF) transmitter,

Received July 19, 2005; revised Jan. 17, 2006; accepted Jan. 17, 2006.

This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan Grant-in-Aid for Scientific Research (K.I., S.K., S.S.), the Core Research for Evolutional Science and Technology (CREST), the Japan Science and Technology Agency (S.K.), and the Wellcome Trust (D.A.R.). We thank C. E. Jahr, F. Saitow, and K. Yoshioka for comments on a draft of this manuscript; K. Shimamoto for the generous gift of TBOA; and C. Kato and H. Nomura for technical assistance for histological experiments.

Correspondence should be addressed to either of the following: Keiji Imoto, National Institute for Physiological Sciences, 5-1 Higashi-yama, Myodaiji-cho, Okazaki 444-8787, Japan, E-mail: keiji@nips.ac.jp; or Shiro Konishi, Waseda-Olympus Bioscience Research Institute, Helios, 05-01/02, 11 Biopolis Way, 138667 Singapore, E-mail: skonishi@waseda.jp.

DOI:10.1523/JNEUROSCI.4894-05.2006

Copyright © 2006 Society for Neuroscience 0270-6474/06/262278-12\$15.00/0

which produces powerful excitation in Purkinje cells (PCs), suppresses inhibitory GABAergic transmission between interneurons and PCs after activation of AMPA receptors (AMPA) (Satake et al., 2000). Both synaptically released transmitter and an exogenous GluR agonist, AMPA, suppress GABA release at basket cell (BC)-PC inhibitory synapses via a presynaptic mechanism that is associated with an AMPAR- and $G_{i/o}$ -protein-dependent pathway, leading to inhibition of presynaptic Ca^{2+} channels (Satake et al., 2004; Rusakov et al., 2005). However, two important issues have yet to be resolved. First, does the neurotransmitter released by the CF input directly act on presynaptic AMPARs in interneuron axon terminals or does it recruit other cellular and molecular components? Second, how does the CF transmitter reach the target AMPARs? Because the CF-PC synapse is surrounded by Bergmann glia (BG) (Iino et al., 2001; Castejón et al., 2002; Grosche et al., 2002) and because PC synapses are enriched with glial and neuronal glutamate transporters (Danbolt, 2001), the uptake machinery might prevent diffusion of the CF transmitter from the synaptic cleft. However, there is another possibility that the CF transmitter can gain access to remote AMPARs: it has been demonstrated recently that AMPARs on BG sense glutamate liberated from ectopic release sites outside the synaptic cleft of CF-PC connections (Matsui and Jahr, 2003, 2004).

We therefore aimed at examining the following: (1) the locus and (2) the subtype of the target AMPARs responsible for GABA release inhibition, and (3) the way the CF neurotransmitter reaches these receptors. Here, we found that the identity of AMPARs mediating the CF-dependent GABA release inhibition differs from that of AMPARs involved in the postsynaptic excitation or activation of BG. Our data also indicate the following: (1) the CF-/AMPA-mediated GABA release inhibition is independent of retrograde messengers and glia-neuron signaling pathways; (2) the CF transmitter diffuses away from its release sites, escaping from glutamate transporters and reaching “presynaptic” GluR2/3-AMPA receptors on BC axon terminals; and (3) GluR2/3-containing AMPARs are detected at axosomatic GABAergic synapses on PCs at light and electron microscopy levels. The results, therefore, shed light on the important principles of target mechanism specificity of the fast excitatory neurotransmitter glutamate, which acts on multiple AMPAR subtypes beyond the immediate synapse.

Materials and Methods

Cerebellar slices and electrophysiological recording. Sagittal cerebellar slices (250 μ m thick) were prepared from Wistar rats (11–18 d of age) as described previously (Saitow et al., 2000). Animals were anesthetized by inhalation of halothane and decapitated in accordance with the institute guidelines. Immediately isolated cerebella were cut by a vibratome (Leica, Nussloch, Germany) in ice-cold saline (Saitow et al., 2000). After incubation with oxygenated saline at room temperature for at least 1 h, the slices were transferred to a recording chamber mounted on the microscope stage and continuously superfused with an artificial CSF (ACSF) that contained the following (in mM): 138.6 NaCl, 3.35 KCl, 2.5 $CaCl_2$, 1.0 $MgCl_2$, 21.0 $NaHCO_3$, 0.6 NaH_2PO_4 , and 10.0 glucose (bubbled with 95% O_2 and 5% CO_2 to maintain pH 7.4 at a room temperature). AMPAR currents were recorded in some experiments in a Na^+ -free, 10 mM Ca^{2+} -ACSF containing the following (in mM): 140 *N*-methyl-D-glucamine, 10 $CaCl_2$, 10 glucose, and 10 HEPES, pH adjusted to 7.4 by HCl (bubbled with 100% O_2) (Iino et al., 2001). Flow rate was 0.5–1.0 ml/min, and all experiments were performed at room temperature. Synaptic responses and GluR agonist-induced currents were recorded from PCs, BCs, and BG by the whole-cell voltage-clamp technique. Membrane currents were held at -80 to -20 mV through a voltage-clamp amplifier (EPC-10; HEKA Elektronik, Lambrecht, Ger-

many) via patch-clamp electrodes (resistance, 2–5 M Ω) filled with an internal solution that contained the following (in mM): 150.0 cesium methanesulfonate, 5.0 KCl, 0.1 K-EGTA, 5.0 Na-HEPES, 3.0 Mg-ATP, 0.4 Na-GTP, pH adjusted to 7.4.

CF- and parallel fiber (PF)-mediated EPSCs and BC-mediated IPSCs were evoked by focal stimulation (single pulse of 5 V for 200 μ s) through ACSF-filled glass electrodes placed in the molecular and granular layers close to the PC soma. CF- and PF-EPSCs were distinguished as described previously (Mitoma and Konishi, 1999). For characterization of AMPAR-induced currents in BCs and BG, AMPA (5 mM) was applied by iontophoresis (Mitoma and Konishi, 1999) in the presence of 10 μ M cyclothiazide (Tocris Cookson, Bristol, UK) to minimize AMPAR desensitization (Partin et al., 1993). Morphology of BG and BC was examined by intracellular staining with biocytin (5 mg/ml; Sigma-Aldrich, St. Louis, MO) during whole-cell recordings. Miniature IPSCs (mIPSCs) were recorded from PCs in ACSF containing 0.5 μ M tetrodotoxin (TTX; Sankyo, Tokyo, Japan) and analyzed using the Mini Analysis program (Synaptosoft, Decatur, GA). Miniature currents were distinguished from baseline noises as described previously (Satake et al., 2000). Spontaneously occurring IPSCs were recorded in the absence of TTX during a constant period of 1–15 s to analyze their frequency and amplitude distributions.

Drug application. All drugs used in this study were bath applied unless otherwise stated. The sources of drugs were as follows: (RS)-AMPA, γ -D-glutamylglycine (γ -DGG), ($\pm$)-4-(4-aminophenyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methylenedioxyphthalazine (SYM2206), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from Tocris Cookson; bromohomoibotenic acid [($\pm$)-Br-HIBO], 1-naphthyl acetyl spermine (NASPM), 8-cyclopentyl-1,3-dimethylxanthine (CPT), and reactive blue-2 (RB-2) were obtained from Sigma-Aldrich; philanthoxin-433 (PhTX) was obtained from RBI (Natick, MA); and thapsigargin and dextran were obtained from Wako Pure Chemical Industries (Osaka, Japan). DL-threo- β -benzyloxyaspartic acid (TBOA) was kindly provided by K. Shimamoto (Suntory Institute for Bioorganic Research, Osaka, Japan). The effects of PhTX were examined after application of PhTX was initiated with AMPA (0.5 μ M) for at least 3 min and then outlasted the AMPA application for 10–15 min. Dextran (40 kDa) was added to the ACSF at a concentration of 5% w/w, and this solution was diluted isosmotically with distilled water (Savtchenko and Rusakov, 2005).

Data analysis. Electrophysiological data were obtained from independent determinations in individual cells in different slices except for comparing the effects of a different drug on CF-induced inhibition of IPSCs and AMPA-induced inward currents in the same cells. The statistical significance of differences was examined by one-way ANOVA or Tukey–Kramer’s parametric multiple comparison test. Differences giving *p* values <0.05 were judged to be significant.

Double-immunofluorescence staining for light microscopy. Wistar rats (10–18 and 53–58 d of age) were deeply anesthetized with diethyl ether and transcardially perfused with 0.1 M PBS, pH 7.2 at 37°C, containing 50 U/ml heparin sodium (Novo Nordisk, Bagsvaerd, Denmark) followed by an ice-cold Zamboni’s fixative (Stefanini et al., 1967). Cerebella were removed and postfixed in the same fixative for at least 2 h. After washing three times with 0.1 M phosphate buffer (PB; pH 7.2), the fixed tissues were immersed in 0.1 M PB containing 20% sucrose at 4°C overnight for cryoprotection, embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen in a mixture of dry ice and acetone. Frozen sections of the cerebella were cut sagittally on a freezing microtome at 10 μ m and mounted on glass slides.

For double-immunofluorescence staining of 65 kDa isoform of glutamate decarboxylase (GAD65) and either AMPARs or vesicular glutamate transporter 2 (VGLUT2), sections were blocked with 5% normal goat serum in 0.1 M PBS containing 0.2% Triton X-100 for 30 min at room temperature and then incubated for at least 2 d at 4°C with a mixture of mouse monoclonal anti-GAD65 (1:400; Chemicon, Temecula, CA) and each of the following primary antibodies: rabbit polyclonal anti-GluR1, 2, 4 (1:40), rabbit polyclonal anti-GluR2/3 (1:50), or guinea pig polyclonal anti-VGLUT2 (1:1000), all obtained from Chemicon, diluted in 0.1 M PBS containing 0.2% Triton X-100. In some experiments, the sections were treated with pepsin or trypsin before incubation with the

primary antibodies (Watanabe et al., 1998; Yamada et al., 2001). After several rinses in 0.1 M PBS, sections were incubated with biotinylated goat anti-rabbit IgG (1:100; Vector Laboratories, Burlingame, CA) for 1 h at room temperature, rinsed again, and incubated with fluorescein isothiocyanate-conjugated streptavidin (1:100; Jackson ImmunoResearch, West Grove, PA) for 1 h to visualize GluRs. Then, sections were rinsed and incubated with rhodamine-conjugated goat anti-mouse IgG (1:100; Chemicon) to visualize GAD65. Double-immunofluorescence staining of AMPARs and synaptophysin were similarly done using mouse monoclonal anti-synaptophysin antibody (1:1000; Chemicon) instead of anti-GAD65. Double-immunofluorescence staining of GAD65 and VGLUT2 were similarly done using biotinylated goat anti-guinea pig IgG (1:500; Jackson ImmunoResearch) and Cy5-conjugated streptavidin (1:1000; Jackson ImmunoResearch). We confirmed no cross-reactivity between each of the anti-GluR antibodies and rhodamine-conjugated goat anti-mouse IgG between anti-GAD65 or anti-synaptophysin antibody and biotinylated goat anti-rabbit IgG and between anti-VGLUT2 antibody and rhodamine-conjugated goat anti-mouse IgG. After rinsing several times, sections were coverslipped and observed with a confocal laser scanning microscopy (Bio-Rad, Hemel Hempstead, UK).

Immunoperoxidase staining for electron microscopy. Wistar rats (8 weeks of age) were anesthetized with an intraperitoneal injection of 7% chloral hydrate and perfused with a solution of normal saline, followed by a fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in a 0.1 M PB, pH 7.4. The cerebellum was removed, and sections were obtained through the rostrocaudal extent of the cerebellum at 100 μ m thickness using a vibratome (Oxford Instruments, Oxford, UK). Free-floating sections were washed in three changes of 0.1 M PBS and incubated for 1 h in a solution of 5% normal goat serum. They were then incubated overnight with a rabbit polyclonal anti-GluR2/3 (1:200; Chemicon). The sections were washed in three changes of PBS and incubated for 1 h in a biotinylated goat anti-rabbit IgG (1:200; Vector Laboratories). After several rinses with PBS, the sections were reacted for 1 h with an avidin-biotinylated horseradish peroxidase complex. The immunoreaction was visualized by treatment for 5 min in 0.05% of 3,3'-diaminobenzidine tetrahydrochloride solution in Tris buffer containing 0.05% hydrogen peroxide. The chemical reaction was stopped with several washes by Tris buffer and PBS.

Electron microscopy was performed by subdissecting the immunostained sections into smaller rectangular blocks that included the molecular layer and the granule cell layer. The blocks were postfixed in 1% osmium tetroxide in 0.1 M PB for 1 h and dehydrated in an ascending series of ethanol and acetone. The blocks were infiltrated with resin and then embedded flat in fresh resin between transparent sheets at 60°C for 24 h in an oven. Thin sections (80–90 nm) were then obtained from the first 5 μ m of the sections, mounted on copper grids coated with Formvar, and stained with lead citrate. They were observed using a Philips EM208 transmission electron microscope (Philips, Eindhoven, Netherlands).

Results

CF/AMPA-mediated inhibition of GABA release is independent of postsynaptic excitation

Repetitive stimulation of the CF input inhibits GABAergic transmission between BCs and PCs by an AMPAR-dependent presynaptic mechanism (Satake et al., 2000, 2004). This inhibitory action appeared synapse specific, because stimulation of PF inputs had no effect (Satake et al., 2000; Rusakov et al., 2005). First, using a subunit-selective AMPAR agonist, Br-HIBO, we tested whether the CF-induced inhibition of GABAergic transmission depends on selective subunit compositions of AMPARs. This compound has been reported to exhibit a differential selectivity to distinct GluR subunit compositions: its binding potency is high (EC_{50} , ~ 5 μ M) for GluR2 channels, intermediate (EC_{50} , ~ 80 μ M) for GluR2/GluR3 channels, and low (EC_{50} , >200 μ M) for GluR3 channels in a *Xenopus* oocyte expression system (Coquelle et al., 2000).

Application of AMPA (0.5 μ M) by perfusion has been shown

Figure 1. Actions of AMPA and Br-HIBO on stimulation-evoked GABA_A-IPSCs, sIPSCs, and the membrane current recorded in PCs. **A**, Successive five IPSCs evoked by stimulation were recorded from a PC before (control) and after bath application of either AMPA (0.5 μ M) or Br-HIBO (5 μ M). A dotted line indicates baseline holding current at -30 mV. **B**, AMPA (0.5 μ M) and Br-HIBO (5 μ M), applied during the period indicated by horizontal bars, induced inward currents (downward deflections) and increased the frequency of sIPSCs (upward deflections) in a PC. **C**, Summary of the GABA_A-IPSC inhibition (open bars) and inward current response (closed bars) produced in PCs by AMPA and Br-HIBO applications (each, 5 min; $n = 9$) and blockade of the both responses by SYM2206 (20 μ M). SYM2206 was pretreated before application of either AMPA ($n = 5$) or Br-HIBO ($n = 6$). Error bars represent SEM. * $p < 0.05$, *** $p < 0.001$.

to produce three distinct actions (Satake et al., 2000, 2004): (1) reduction of the amplitude of stimulation-evoked IPSCs (eIPSCs); (2) increase in the frequency of spontaneously occurring IPSCs (sIPSCs); and (3) postsynaptic inward currents (170 ± 43 pA; $n = 7$) in PCs voltage clamped at -30 mV (Fig. 1A,B). In contrast, the ibotenic acid analog Br-HIBO (5 μ M) did not significantly reduce the eIPSC amplitude, although it produced inward currents in PCs with the average amplitude (176 ± 62 pA; $n = 6$) similar to that following application of 0.5 μ M AMPA (Fig. 1A,B; see C for a summary). An AMPAR-specific antagonist, SYM2206 (20 μ M) (Li et al., 1999; Pei et al., 1999), completely abolished both AMPA- and Br-HIBO-induced currents (Fig. 1C), indicating that selective activation of AMPARs is responsible for inducing the current responses in PCs.

Another difference between the two AMPAR agonists was that their potencies in increasing the sIPSC frequency were significantly different, Br-HIBO being less potent than AMPA ($p <$

Figure 2. Presynaptic actions of the two GluR agonists recorded in PCs and BCs. *A, B*, Successive two traces (including sIPSCs) recorded from a PC before (control) and during bath application of either AMPA (0.5 μ M; *A*) or Br-HIBO (5 μ M; *B*). *C*, Summary of the increases in sIPSC frequency in PCs induced by AMPA (0.5 μ M; $n = 7$) and Br-HIBO (5 μ M; $n = 6$). *D*, An example of recordings for membrane currents and spontaneous synaptic currents from a BC. Each of the two agonists was bath-applied during the period indicated by a horizontal bar. AMPA (0.5 μ M) induced an inward current (downward slow deflection) and increases in spontaneous synaptic responses, whereas Br-HIBO (5 μ M) produced only moderate actions on the same BC. Error bars represent SEM.

0.05) (Fig. 2*A–C*), which suggests that there might be different subunit compositions between postsynaptic PC-AMPA receptors and somatodendritic BC-AMPA receptors, the latter contributing to excitation in BCs, thereby leading to the increase in sIPSCs in PCs. Although the two AMPAR agonists exhibited a similar potency for the postsynaptic excitatory action on PCs, Br-HIBO was also less potent than AMPA in inducing inward currents in presynaptic BCs (Fig. 2*D*), which might reflect the heterogeneity of GluRs at synaptic and extrasynaptic localizations in cerebellar interneurons as demonstrated by Liu and Cull-Candy (2000, 2002).

The results clearly indicate that AMPAR-mediated inhibition of GABA release from interneurons (i.e., disinhibition of the PC) and postsynaptic excitation in PCs could be pharmacologically distinguished, thus supporting the hypothesis that CF- and AMPAR-mediated presynaptic inhibition of GABAergic transmission at BC-PC synapses does not depend on the AMPAR-mediated postsynaptic action (Satake et al., 2004). The data further point to the Br-HIBO-insensitive, GluR3-enriched subtype of AMPARs underlying the CF-induced disinhibition.

CF-/AMPA-mediated disinhibition depends on presynaptic but not BG AMPARs

The above result suggests two possible sites of AMPARs for CF-/AMPA-dependent disinhibition: one at the presynaptic terminal of GABAergic interneuron BCs and the other at BG that might activate glia-neuron signaling to suppress GABA release from BCs. To examine the two possibilities, we compared the properties of AMPARs on BG and BCs. It has been shown previously that BG cells selectively express a Ca^{2+} -permeable, rectifying AMPAR subtype that lacks GluR2 subunits (Iino et al., 2001). Indeed, AMPA-induced currents recorded in BG exhibited properties characteristic of this AMPAR subtype: namely, strong inward rectification and high Ca^{2+} permeability, whereas AMPA-

induced responses recorded in BC showed an almost linear I - V relationship and very weak Ca^{2+} permeability (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), suggesting that exogenous AMPA-induced currents in BG and BC resulted from activation of AMPARs with different subunit compositions. In support of this notion, PhTX (10 μ M), a specific blocker of Ca^{2+} -permeable AMPARs (Tóth and McBain, 1998), strongly suppressed the AMPA-induced currents recorded from BG in both control and Na^+ -deficient (10 mM Ca^{2+}) solutions, whereas it caused a moderate blocking action on the AMPA currents recorded in BCs (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). The results suggest that the subunit compositions of GluRs sensitive to exogenous AMPA in BCs and BG are considerably different in terms of their Ca^{2+} permeability.

Based on this striking property of PhTX, we next aimed to determine whether the Ca^{2+} -permeable AMPAR in BG could contribute to the CF-induced inhibition of GABAergic transmission. PhTX (10 μ M) affected neither the magnitude of GABAergic IPSC inhibition nor

the amplitude of EPSCs produced in PCs in response to repeated activation of CF input (Fig. 3*A–C*). Another inhibitor of Ca^{2+} -permeable AMPARs, NASPM (100 μ M) (Matsui and Jahr, 2003) also caused no significant effect on the CF-induced suppression of GABA release (Fig. 2*C*). Conversely, the broad-spectrum AMPAR antagonist SYM2206 (20 μ M) markedly suppressed the inhibitory action of CF input on GABAergic transmission (Fig. 3*C*). Moreover, PhTX did not influence the inhibition of eIPSCs induced not only by the synaptically released CF transmitter but also by exogenous AMPA application (Fig. 3*C*). Although AMPA increased the frequency of sIPSCs and TTX-insensitive mIPSCs (Satake et al., 2004), these responses were also not affected by PhTX (Fig. 3*D*). These data rule out Ca^{2+} -permeable perisynaptic glial AMPARs from the CF-induced inhibition of GABA release. Together with the effect of Br-HIBO (Fig. 1), the PhTX experiments showed that the Ca^{2+} -impermeable AMPAR, most likely composed of GluR2 and GluR3 subunits, mediates the CF-induced presynaptic inhibition of GABAergic transmission at BC-PC synapses.

It has been reported recently that glutamatergic input activates GluRs on hippocampal astrocytes to induce ATP release and thereby its metabolites (presumably adenosine) causes presynaptic inhibition of GABAergic transmission via activation of A1 and/or P2Y purinoceptors at inhibitory synapses (Zhang et al., 2003). The GluR activation is also proposed to liberate diffusible messengers from astrocytes by triggering Ca^{2+} release from their intracellular Ca^{2+} stores (Kirischuk et al., 1999; Coco et al., 2003; Liu et al., 2004). Thus, we examined possible involvements of adenosine A1/P2Y purinoceptors and intracellular Ca^{2+} signaling pathway in the CF-/AMPA-mediated disinhibition. First, a mixture of purinoceptor antagonists, CPT (0.5 μ M) and RB-2 (5 μ M) (Zhang et al., 2003) did not affect the CF-induced disinhibition (Fig. 3*C*). A more selective adenosine A1 antagonist, DPCPX

Figure 3. Tests for determining roles of Ca^{2+} -permeable and impermeable AMPARs in the CF- and AMPAR-induced disinhibition. **A**, Effects of PhTX on the CF stimulation-induced inhibition of eIPSCs in a single PC. Successive five traces of control (left) and CF-conditioned IPSCs (right) recorded in the absence (top) and the presence of $10 \mu\text{M}$ PhTX (bottom). IPSCs were evoked every 15 s by constant paired-pulse (arrowhead; the first IPSC peak) and conditioning stimulation (5 Hz for 5 pulses) to the CF input that preceded IPSC for 1 s was intermittently interposed to see the inhibitory action of CF on GABA release. **B**, Averaged traces of each first response for control (gray) and conditioned IPSCs (black) as indicated by arrowheads in **A** displayed on expanded amplitude and time scales were superimposed in normal ACSF (top) and a PhTX-containing ACSF (bottom). **C**, Effects of SYM2206 ($20 \mu\text{M}$), PhTX ($10 \mu\text{M}$), NASPM ($100 \mu\text{M}$), CPT ($0.5 \mu\text{M}$)/RB-2 ($5 \mu\text{M}$) mixture, DPCPX ($10 \mu\text{M}$), or thapsigargin (Thapsi; $1 \mu\text{M}$) on the degree of CF-induced or AMPA-induced ($0.5 \mu\text{M}$) disinhibition. Each column represents the mean $\pm$ SEM ($n = 6-34$). $***p < 0.001$. **D**, Effects of PhTX ($10 \mu\text{M}$) or thapsigargin ($1 \mu\text{M}$) on the AMPA ($0.5 \mu\text{M}$)-induced increases in the frequency of sIPSC and mIPSC. Each column represents the mean $\pm$ SEM ($n = 5-11$). Neither PhTX nor thapsigargin affected significantly the AMPA-induced both responses.

($10 \mu\text{M}$), also did not alter the CF stimulation- and exogenous AMPA-induced inhibition of eIPSCs (Fig. 3C), and DPCPX at this concentration abolished the inhibitory action of adenosine ($300 \mu\text{M}$) on PF-mediated EPSCs in PCs (data not shown). Second, thapsigargin ($1 \mu\text{M}$), an inhibitor of endoplasmic reticulum

Ca^{2+} pumps and Ca^{2+} accumulation in internal stores (Kirischuk et al., 1999), had no significant effects on the extents of CF- and AMPA-induced responses (Fig. 3C,D). The AMPAR-mediated inhibition of eIPSCs was not changed by a CB1 endocannabinoid antagonist, AM251 (Satake et al., 2004), and an inhibitor of cyclooxygenase, indomethacin ($20 \mu\text{M}$) (data not shown). Together, these data argue against the mechanism whereby diffusible messengers, such as ATP/adenosine, endocannabinoids, and cyclooxygenase products (e.g., prostanooids) released by AMPAR activation (and subsequent intracellular Ca^{2+} elevation) from BG contribute to the inhibitory action of CF input.

Immunohistochemical localization of AMPARs in the cerebellar cortex

Although the results from electrophysiological experiments predict the presence of presynaptic AMPARs in the cerebellar cortex, their localization has not been determined precisely by morphological techniques. We therefore performed double immunohistochemical labeling for GluR1–4 subunits and GABA synthesizing enzyme, GAD65, in cerebellar tissue slices prepared from 2- or 8-week-old rats. In agreement with the previous findings (Petrálie and Wentholt, 1992), antibodies against GluR2 and GluR3 showed intense immunolabeling on PC somata and dendrites but not on BG (Fig. 4A,B). An antibody against GluR1 stained BG in the molecular layer and labeling with a GluR4-recognizing antibody showed diffuse immunoreactivity throughout the molecular layer (data not shown), probably reflecting wide-spreading BG processes that might express GluR1/4. A prominent feature was that double labeling for GluR2/3 subunits and GAD65 (Esclapez et al., 1994) was indeed colocalized in perisomatic and pinceau-like processes that surround the cell body of PCs (Fig. 4A,D–F) (a trypsinized preparation from 8-week-old rats; see Materials and Methods). Similar albeit more extensive patterns of colocalized GluR2/3 and GAD65 distributions were observed in preparations without the trypsin treatment (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Beaded double labels for GluR2/3 and GAD65 were also detected on the PC layer and the primary dendrites of PCs in a preparation derived from 2-week-old rats (Fig. 4C) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material); this age matched that used for electrophysiological recordings. Because GAD65 protein is concentrated in the presynaptic structures such as perisomatic boutons (PC pericellular nest), the pinceaux at the base of PC somata and glomeruli in the granular layer (Fig. 4E) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) (Esclapez et al., 1994; Obata et al., 1999), the finding from double immunostaining observed here points to a presynaptic localization of AMPARs in GABAergic BC axon terminals. This possibility was further supported by an observation that immunoreactivity for synaptophysin, a presynaptic marker, is also colocalized with the fine, often beaded labels for GluR2/3 at perisomatic regions of PCs and the pinceau-like processes around the PC body (Fig. 4B,G–I). Furthermore, GluR2/3 labeling appeared to occur in GFP fluorescent-positive nerve terminals of BCs in GAD67-GFP knock-in mice (supplemental Fig. 3, available at www.jneurosci.org as supplemental material) (Tamamaki et al., 2003).

To further determine the location of presynaptic AMPARs at the subsynaptic level, we performed immunoelectron microscopic analysis for GluR2/3 subunits in preparations derived from 8-week-old rats using immunoperoxidase method. Electron-dense deposits of immunoreaction product were local-

Figure 4. Immunohistochemical staining of AMPARs, GAD65, synaptophysin, and VGLUT2 in the cerebellar cortex. **A–C**, Confocal micrographs of double immunofluorescence detected by antibodies for AMPARs (GluR2/3 subunits; green, **A–C**), GAD65 (red, **A, C**), and synaptophysin (red, **B**). Sections were pretreated with trypsin (see Materials and Methods). **A** and **B** were derived from 8-week-old rats, and **C** was derived from a 2-week-old rat. **D–F**, Higher-magnification images of a rectangular area indicated in **A**: GluR2/3 (**D**), GAD65 (**E**), and merged (**F**). **G–I**, Enlarged from the micrograph (**B**) as in **D–F**: GluR2/3 (**G**), synaptophysin (**H**), and merged (**I**). Arrows indicate the pinneau, and arrowheads represent examples for colocalized immunoreaction products. **J, K**, Double immunofluorescence detected by antibodies for GAD65 (red) and VGLUT2 (blue). Asterisks indicate PC somata, and the images were derived from a 2-week-old rat. The area outlined in **J** is enlarged in **K**. ML, Molecular layer; PCL, Purkinje cell layer; GCL, granule cell layer. Scale bars: **A, B, J, K**, 40 μ m; **C**, 20 μ m; **D–I, K**, 10 μ m.

ized in the axon terminals of BCs making symmetrical synaptic contacts with the soma of PCs (Fig. 5*A, B*). Such products were associated with the presynaptic membrane (Fig. 5*B, C*, arrows) and also with some synaptic vesicles distributed preferentially at the perisynaptic regions.

We next examined spatial relationships between BC- and CF-nerve terminals using the vesicular glutamate transporter VGLUT2 as a specific marker of the CF terminal (Hioki et al., 2003). Beaded immunoreactivities for VGLUT2 densely surrounded the PC soma and partially extended to the main shaft of its dendrites in the cerebellar cortex (Fig. 4*J, K*, 2-week-old rat). VGLUT2 staining was often in a close apposition to pericellular GAD65 immunoreactivities, suggesting a possible site of functional interaction between CF and BC terminals. The staining

profile of VGLUT1, a specific marker of the PF terminal, was in a sharp contrast to that of VGLUT2, exclusively distributing to a region of proximal and distal dendrites of PCs in the molecular layer but not to the PC soma (data not shown).

AMPA-mediated inhibition of GABA release depends on diffusion of CF transmitter

The above findings led to the hypothesis that the CF transmitter can gain access to presynaptic AMPARs in BC terminals through extrasynaptic diffusion. Because the concentration of CF transmitter that reaches presynaptic AMPARs in BC terminals should be lower than that within the cleft of CF-PC synapses, we next examined the effects of a competitive low-affinity GluR antagonist, γ -DGG, on the CF-induced EPSCs in PCs and GABA release inhibition at BC-PC synapses. γ -DGG has been demonstrated to inhibit more effectively the action of low-glutamate transient (e.g., released after repeated presynaptic stimulation or diffused from the release site) than high-glutamate transient-induced postsynaptic excitation resulted from direct synaptic connections (Wadiche and Jahr, 2001; Matsushita et al., 2002). As expected, the blocking action of γ -DGG (2 mM) on CF-induced EPSCs became prominent when CF input was repeatedly stimulated (compare the first and successive EPSCs in the presence and absence of γ -DGG) (Fig. 6*A, B*), reflecting the decrease in the CF transmitter amount after repetitive stimulation. γ -DGG markedly suppressed the CF-induced inhibition of eIPSCs (Fig. 6*A, C*), and this suppression was significantly larger than the effect of γ -DGG on the CF-EPSCs determined as total charge transfer of the synaptic response during repetitive conditioning stimulation (Fig. 6*E*). As summarized in Figure 6*F*, the potency of γ -DGG in suppressing the CF disinhibition depended on the frequency of CF stimulation (namely, transmitter concentrations at release

sites), being significantly reduced with changing the frequency from 5 to 50 Hz. These results suggested that the CF transmitter reaches presynaptic AMPARs via extracellular diffusion from the relatively remote release site rather than through direct synaptic connections between CFs and BCs (but see Sugihara et al., 1999; Castejón et al., 2001; Jörntell and Ekerot, 2003).

This was further examined in the experiments in which we aimed to retard diffusion of the CF transmitter in the extracellular space using inert dextran molecules (40 kDa). The substantial viscosity increase caused by 5% dextran solution has been reported to retard diffusion coefficient of neurotransmitters in the extracellular space (Rusakov and Kullmann, 1998; Piet et al., 2004) by 20–40% (Nielsen et al., 2004; Savtchenko and Rusakov, 2005). Addition of dextran (5% w/w) into perfusion solution

Figure 5. Electron micrographs showing immunoreactivity for presynaptic AMPARs (GluR2/3 subunits) in the cerebellar cortex. Sections derived from 8-week-old rats were processed by the pre-embedding immunoperoxidase method. The area outlined in *A* is enlarged in *B*. Arrows point to immunoreaction products associated with presynaptic membrane and synaptic vesicles, and arrowheads indicate synaptic contacts between a PC soma and an axon terminal of a presumed BC. N, Nucleus; A, axon terminal; S, Purkinje cell soma. Scale bars: *A*, 1 μ m; *B*, *C*, 200 nm.

caused a significant suppression of the CF-induced eIPSC inhibition ($p < 0.05$) (Fig. 6*D*), whereas inclusion of dextran per se caused no significant change in the magnitude of CF stimulation-induced EPSC charge ($115 \pm 22\%$; $n = 9$; $p > 0.4$). This is consistent with the notion that, by increasing the dwell time of glutamate molecules in the proximity of glutamate transporters, diffusion retardation should increase the efficiency of uptake and thus prevent further escape of glutamate toward BC axon terminals. In contrast to the blocking action of γ -DGG, the extent of this effect did not depend on the frequency of CF stimulation (Fig. 6*F*).

CF transmitter partly escapes from glutamate transporters

If the CF transmitter could diffuse from its release sites, excitatory amino acid transporters (EAATs) would regulate this diffusion. To test this possibility, we examined the effects of an EAAT blocker, TBOA (Shimamoto et al., 1998), on CF- and PF-mediated glutamatergic transmission as well as on the CF disinhibition. First, bath application of TBOA (30 μ M) prolonged the time course of CF-EPSC (Fig. 7*A*): the decay time constant (τ) of CF-EPSC increased from 7.3 ± 0.7 to 13.3 ± 2.0 ms ($n = 7$; $p < 0.05$) (Takayasu et al., 2004). Although TBOA (30 μ M) also appeared to prolong PF-EPSC (Fig. 7*A*), this effect was not significant: the τ values of PF-EPSC were 7.7 ± 0.5 and 9.7 ± 1.0 ms in the absence and presence of TBOA, respectively ($n = 7$; $p > 0.1$). Thus, the activity of EAATs likely shapes the glutamatergic EPSC more prominently at CF-PC synapses than PF-PC synapses, depending on the amount of excitatory transmitters at individual release sites.

Second, TBOA significantly augmented the CF-induced inhibition of GABAergic IPSCs (Fig. 7*B, C*), and this effect was markedly suppressed by SYM2206 (Fig. 7*C*). However, TBOA increased only the magnitude but not the duration of CF-induced IPSC inhibition (Fig. 7*D*). The decay of CF-inhibitory action was described by a single exponential, indicating that a single mechanism underlies this inhibition (Satake et al., 2000). The fact that the decay time constant was not altered by TBOA suggests that the duration of CF inhibition is determined by the process downstream of AMPAR activation [e.g., G-protein-dependent pathways that link AMPAR-mediated inhibition of presynaptic Ca^{2+} channels (Satake et al., 2004)] rather than transporter-mediated clearance of the CF transmitter.

Finally, the fact that the strength of GABAergic transmission at BC-PC inhibitory synapses could be affected by extrasynaptic diffusion of CF transmitter suggests that blocking EAATs by TBOA could mimic the inhibitory action of CF stimulation. This possibility was tested in the experiments illustrated in Figure 8. A high concentration of TBOA (200 μ M) caused a profound inhibitory action on stimulation-evoked IPSCs (Fig. 8*A*); the eIPSC amplitude decreased to $42.2 \pm 6.4\%$ of baseline IPSCs ($n = 5$), and this effect recovered completely within 15 min after termination of TBOA treatment (Fig. 8*C*). In contrast to exogenous AMPA (0.5 μ M)-induced inhibition, the TBOA-induced suppression of IPSCs was not associated with changes in the membrane current of PCs (Fig. 8*E*), thus indicating that TBOA inhibited GABAergic IPSCs without relying on the postsynaptic current response in PCs. This inhibitory action of TBOA was relatively selective to BC-PC inhibitory synapses, because TBOA (100 μ M) suppressed BC-PC IPSCs and PF-EPSCs to 48.0 ± 16.2 ($n = 10$) and $73.8 \pm 14.8\%$ ($n = 6$), respectively ($p < 0.05$, IPSC vs PF-EPSC suppression by TBOA). More importantly, the TBOA (200 μ M)-induced inhibition of eIPSCs was significantly suppressed by the AMPAR antagonist SYM2206 (100 μ M) (Fig.

Figure 6. Effects of γ -DGG and dextran on CF-induced disinhibition. **A**, Control (left) and CF-conditioned IPSCs (right) in a PC were recorded as in Figure 3A before (left) and during (right) application of 2 mM γ -DGG. **B**, Averaged traces of the first and the second CF-EPSCs recorded before (gray) and during (black) γ -DGG application were superimposed. **C, D**, Averaged traces of each first response for control (gray) and conditioned IPSCs (black) displayed on expanded amplitude, and time scales were superimposed in normal ACSF (left) and either a 2 mM γ -DGG-containing (**C**) or 5% dextran-containing (**D**) ACSF (right). **E**, Comparison of the effects of γ -DGG on the CF-induced disinhibition (vertical axis) and the CF-EPSC (horizontal axis). Changes in CF-EPSCs were measured as the amount of charge transfer. Note that γ -DGG was more effective in suppressing the CF disinhibition than the CF-EPSC. **F**, Effects of γ -DGG (2 mM) and dextran (5% w/w) on the degrees of CF disinhibition induced by conditioning repeated CF stimulation for 1 s at 5 or 50 Hz. Either γ -DGG-containing (black column) or dextran-containing (gray column) ACSF was applied by perfusion. Each column represents the mean $\pm$ SEM ($n = 7$ –16). * $p < 0.05$, *** $p < 0.001$.

8 B); the eIPSC amplitude decreased by TBOA to $73.7 \pm 7.0\%$ in the presence of SYM2206 (Fig. 8, compare *C* and *D*) ($n = 5$; $p < 0.05$). Plausible explanations for the inhibitory action of TBOA on GABAergic IPSCs are as follows: (1) after blockade of glutamate uptake machinery, the concentration of spontaneously released excitatory transmitter (presumably glutamate) could become sufficient to induce crosstalk to presynaptic AMPARs in BC axon/terminals; (2) this crosstalk is independent of postsynaptic depolarization in PCs, probably because excitatory transmitter accumulated by TBOA in the cleft might desensitize the GluRs in PCs (Takayasu et al., 2004); and (3) partial suppression of the TBOA action by the AMPAR antagonist could be attributed to involvements of other glutamate receptors that modulate GABA release at BC-PC synapses (Huang and Bordey, 2004).

Discussion

It is becoming clear that glutamate mediates not only excitation in the postsynaptic target but also presynaptic regulation of neu-

rotransmitter release through homosynaptic and heterosynaptic interactions (Min et al., 1999; Satake et al., 2000; Schmitz et al., 2001; Huang and Bordey, 2004). More recently, glutamate-induced retrograde signaling involving diffusible messengers liberated by postsynaptic neurons (Alger, 2002; Diana and Marty, 2004; Brenowitz and Regehr, 2005) and/or neuron-glia signaling pathways (Zhang et al., 2003) has also been proposed to regulate neurotransmission at many central synapses. Although both ionotropic and metabotropic GluRs have been implicated in the control of synaptic strength, the underlying mechanisms are not precisely defined. Here, we examined the CF transmitter-mediated, AMPAR-dependent presynaptic inhibition of GABAergic transmission at cerebellar inhibitory synapses. Our findings strongly suggest that AMPARs, which occur in the presynaptic terminals of cerebellar interneurons, can directly mediate suppression of GABA release after activation by the CF excitatory transmitter via extrasynaptic diffusion. A line of evidence we obtained is as follows: (1) CF stimulation can suppress GABAergic transmission at the BC-PC connection without requiring retrograde signaling; (2) the glia-to-neuron signaling resulting from activation of adjacent BG (Matsui and Jahr, 2003, 2004) and other glial cells (Lin et al., 2005) is not involved in the inhibitory action of CF input; and (3) the CF transmitter can reach remote AMPARs, most likely GluR2/GluR3 heteromers, in the BC axon/terminals by escaping from the uptake capacity provided by glial and neuronal glutamate transporters around CF-PC synaptic contacts. Therefore, crosstalk between the CF and BC inputs, by recruiting local subunit selectivity of presynaptic AMPARs, inhibits GABA release, thereby disinhibiting the PC and strengthening the CF input to the cerebellar output when high throughput is physiologically required in the CF-PC pathway.

CF disinhibition depends on presynaptic GluR2/3-AMPA receptors in BC axon terminals

Using specific pharmacological tools, we accessed the subtype and location of AMPARs involved in the CF inhibitory action. First, Br-HIBO, an AMPAR agonist with a higher selectivity to homomeric GluR2- compared with GluR3-containing AMPARs (Coquelle et al., 2000), was effective in producing a postsynaptic excitatory action in PCs without the inhibitory action on GABAergic transmission, whereas AMPA produced both actions (Fig. 1), indicating that the GluR3 can contribute to the AMPAR-mediated inhibitory action at BC-PC synapses. Br-HIBO was also much less potent than AMPA in inducing presynaptic actions, such as inward currents in BCs and increase in sIPSC frequency via BC excitation (Fig. 2). Cerebellar interneurons have been shown to express heterogeneous GluR subunits at synaptic and

extrasynaptic sites (Liu and Cull-Candy, 2000, 2002), which might explain the differential potency of Br-HIBO for activation of presynaptic BC- and postsynaptic PC-AMPA receptors. The Br-HIBO experiments also suggest that retrograde signaling following the postsynaptic excitation in PCs is not a prerequisite for the function of inhibitory AMPARs (Satake et al., 2004).

Second, PhTX and NASPM, the open-channel blockers of Ca^{2+} -permeable AMPARs lacking GluR2 subunits (Tóth and McBain, 1998; Matsui and Jahr, 2003), did not affect the CF-/AMPA-mediated disinhibition (Fig. 3) but markedly suppressed the AMPAR current in BG (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), implying that the GluR2-lacking, Ca^{2+} -permeable AMPARs in BG are not responsible for the CF-induced GABA release inhibition. The result also makes it unlikely that other glial cells, such as NG2-immunoreactive oligodendrocyte precursor cells, tightly innervated by CFs, could be involved in the CF disinhibition, because Ca^{2+} -permeable AMPARs mediate the CF stimulation-induced inward current in the NG2-positive glial cell (Lin et al., 2005). The observation that the low-affinity GluR antagonist γ -DGG suppressed the CF disinhibition more potently than CF-EPSCs (Fig. 6) could also rule out the involvement of the tight connection between CFs and glial cells in the CF-mediated inhibitory action. The other action caused by activation of presynaptic AMPARs, namely the increase of mIPSC frequency, was also insensitive to PhTX. The AMPAR activation-induced increase in the frequency of mIPSCs recorded from cerebellar interneurons has been reported previously to occur transiently during development of cerebellar circuitry (Bureau and Mulle, 1998).

Third, immunohistochemistry for GluR subunits supports the interpretation derived from the Br-HIBO and PhTX experiments. In agreement with the previous studies suggesting that cerebellar interneurons express GluR2 and GluR3 subunits (Keinänen et al., 1990; Petralia and Wenthold, 1992; Lin and Cull-Candy, 2000, 2002), our data indicated that GluR2/GluR3 staining was colocalized with that of the GABA synthesizing enzyme GAD65 or a presynaptic marker, synaptophysin, in beaded structures around PC bodies, presumably reflecting the BC-derived presynaptic terminals (Fig. 4). This was further supported by electron microscopic immunohistochemistry (Fig. 5). The GluR2/3 immunoreactivity occurred in the presynaptic axon terminals of BC, making symmetrical synapses with the PC soma. Because the immunoreaction product was often in close association with the presynaptic membrane and synaptic vesicles at perisynaptic regions, it is highly likely that presynaptic AMPARs interact with P/Q -type Ca^{2+} channels around the active zone to modulate the Ca^{2+} channel activity for suppressing GABA release (Satake et al., 2004; Rusakov et al., 2005). P/Q -type Ca^{2+} channels have been shown to play a major part in action

Figure 7. Effects of glutamate uptake blockade on CF- and PF-EPSCs and the CF-induced disinhibition. **A**, Successive superimposed CF-EPSCs (top) and PF-EPSCs (bottom) in PCs recorded in normal (left) and 30 μM TBOA-containing ACSF (middle) and averaged traces of the first responses before (gray) and during TBOA application (black). **B**, Averaged traces for control (gray) and CF-conditioned IPSCs (black) were superimposed in normal ACSF (left) and a 30 μM TBOA-containing ACSF (right). Conditioning stimulation to CF input was the same as in Figure 3A. **C**, TBOA-induced enhancement of the CF disinhibition and its suppression by SYM2206. The concentrations of drugs applied by perfusion are indicated under columns. Each column represents the mean $\pm$ SEM ($n = 8$ –16). $***p < 0.001$. **D**, Effects of TBOA on the time course of CF disinhibition. The degrees of IPSC inhibition were plotted (in a log scale) on intervals (Δt) between the end of CF-conditioning stimulation (50 Hz for 40 pulses) and test stimuli to evoke IPSCs in the absence (open circles) or presence of 30 μM TBOA (closed circles). Each point represents the mean $\pm$ SEM ($n = 6$ –8).

potential-evoked $[\text{Ca}^{2+}]$ rises in BC axonal boutons (Forti et al., 2000).

The presence of GluR2/3-AMPA receptors in the presynaptic terminal is consonant with the possibility raised recently by Schenk et al. (2003, 2005) that GluR1/2/3-AMPA receptors are delivered through synaptic vesicle recycling to the axon plasma membrane and involved in regulation of synaptic vesicle redistribution in neurotransmitter pools. Presynaptic AMPAR activation has been shown to suppress the Ca^{2+} channel activity, thereby inhibiting GABA release from inhibitory interneuron terminals (Satake et al., 2004; Rusakov et al., 2005), although it should be further examined whether AMPARs regulate the release machinery directly via ion flux or indirectly via signaling pathways (Wang et al., 1997; Hayashi et al., 1999; Satake et al., 2004; Takago et al., 2005).

CF transmitter reaches presynaptic AMPARs via extrasynaptic diffusion from release sites

How could the CF transmitter reach the presynaptic AMPARs in BCs? This question was examined by the following experiments (Figs. 6, 7). First, the blocking action of low-affinity GluR antagonist γ -DGG (Wadiche and Jahr, 2001; Matsushita et al., 2002) was far more potent on the CF disinhibition than the postsynaptic excitation (CF-EPSC), and the γ -DGG-induced suppression of CF action on GABA release was reduced by increasing the frequency of CF stimulation. Second, retarding extracellular dif-

Figure 8. Effects of a high concentration of TBOA on stimulation-evoked IPSCs recorded from a single PC. **A, B**, Successive five traces of eIPSCs were superimposed before (control) and during application of TBOA ($200\ \mu\text{M}$) in normal ACSF (**A**) and in the presence of SYM2206 ($100\ \mu\text{M}$) (**B**). **C, D**, Time course of the inhibitory action of TBOA on IPSCs evoked every 15 s in normal ACSF (**C**) and SYM2206-containing ACSF (**D**). TBOA ($200\ \mu\text{M}$) and SYM2206 ($100\ \mu\text{M}$) were applied by perfusion during the period indicated by horizontal bars (black and gray, respectively). The IPSC amplitude was expressed as percentage of control responses determined before TBOA application. Each point represents the mean $\pm$ SEM ($n = 5-6$). **E**, Effects of AMPA ($0.5\ \mu\text{M}$) and TBOA ($200\ \mu\text{M}$) on the membrane current of a PC. Each drug was applied by perfusion during the period indicated by a horizontal bar. Note that AMPA induced an inward current, whereas TBOA did not cause any discernible current.

fusion of neurotransmitters by dextran (Rusakov and Kullmann, 1998; Nielsen et al., 2004; Piet et al., 2004; Savtchenko and Rusakov, 2005) suppressed the CF-induced inhibition of GABA release. Conversely, blockade of glutamate transporters by TBOA (Shimamoto et al., 1998) augmented the inhibitory action of CF input. These observations render strong support for the hypothesis that the CF transmitter can diffuse from the release sites of the CF synaptic input and gain access to presynaptic AMPARs in the BC axon/terminals, thereby directly inhibiting GABA release at the BC-PC synapse.

Glial and neuronal glutamate transporters are essential for terminating actions of the CF transmitter (Takayasu et al., 2005). If the CF transmitter escaped from the release site, blocking the transporter activity would have enhanced the CF-dependent inhibition of GABA release or even mimic the inhibitory action of CF input. Indeed, the EAAT blocker TBOA augmented the CF disinhibition, and TBOA itself at a high concentration suppressed BC-PC IPSCs more effectively than PF-EPSCs (Figs. 7, 8), suggesting that the CF transmitter liberated into the CF-PC synaptic cleft could reach the presynaptic AMPARs in BC to inhibit GABA release.

The alternative explanations for the inhibitory action of TBOA on GABAergic synapses are that the CF transmitter can

diffuse from ectopic release sites of CFs, because a considerable amount of CF transmitter derived from ectopic release sites is sensed by AMPARs in BG (Matsui and Jahr, 2003, 2004), and that the ambient glutamate level rises after EAAT inhibition, thereby leading to activation of GluRs at BC terminals. Alternatively, there is morphological evidence that supports direct synaptic interactions between CF and BC inputs: (1) a study by single axon tracing of CFs shows that some tendril fibers extended from CF terminal arborizations surround the PC soma (Sugihara et al., 1999); (2) an electron microscopic study describes axosomatic contacts that occur between CFs with BCs (Castejón et al., 2001); and (3) electrophysiological studies have shown a direct functional connection between CFs and interneurons (Jörntell and Ekerot, 2003). The results from our γ -DGG and dextran experiments are, however, not in favor of tight CF-BC connections for the inhibitory action of CFs, but rather suggest that the CF transmitter reaches the presynaptic AMPARs in BCs via diffusion from the release site at CF-PC connections.

Because the PF passes through the PC dendritic tree in the molecular layer (Palay and Chan-Palay, 1974), the excitatory transmitter released from CFs but not from PFs could gain access to the presynaptic BC-AMPA receptors (Rusakov et al., 2005). This notion is supported by our morphological evidence showing a close apposition between VGLUT2-stained CF terminals and GAD65-positive BC terminals in the PC layer (Fig. 4) as well as apposed terminal boutons derived from CFs and BCs on a single PC soma (Rusakov et al., 2005), whereas PF terminals stained by the specific marker VGLUT1 (Hioki et al., 2003) was confined to the molecular layer harboring the shafts and trees of PC dendrites.

One emerging feature from this study is that the AMPAR, classically thought to serve as the excitatory receptor, mediates multiple functions in cerebellar synapses depending on local heterogeneity of subunit compositions. The AMPAR activation caused by the CF input at different spatial domains likely produces distinct actions: (1) postsynaptic excitation on the PC, (2) presynaptic form of plasticity transiently suppressing GABAergic inputs on the single PC, and (3) control of perisynaptic glial cell functions. The presynaptic inhibition of GABAergic transmission after direct activation of AMPARs in interneuron terminals by the neurotransmitter escaping from CF synapses, therefore, provide a likely mechanism gating the excitatory input from the CF pathway to the output neuron in the cerebellum.

References

- Alger BE (2002) Retrograde signaling in the regulation of synaptic transmission: focus on endocannabinoids. *Prog Neurobiol* 68:247–286.
- Brenowitz SD, Regehr WG (2005) Associative short-term synaptic plasticity mediated by endocannabinoids. *Neuron* 45:419–431.
- Bureau I, Mulle C (1998) Potentiation of GABAergic synaptic transmission by AMPA receptors in mouse cerebellar stellate cells: changes during development. *J Physiol (Lond)* 509:817–831.
- Castejón OJ, Castejón HV, Sims P (2001) Light microscopy, confocal laser scanning microscopy, scanning and transmission electron microscopy of cerebellar basket cells. *J Submicrosc Cytol Pathol* 33:23–32.
- Castejón OJ, Dailey ME, Apkarian RP, Castejón HV (2002) Correlative microscopy of cerebellar Bergmann glial cells. *J Submicrosc Cytol Pathol* 34:131–142.
- Coco S, Calegari F, Pravettoni E, Pozzi D, Taverna E, Rosa P, Matteoli M, Verderio C (2003) Storage and release of ATP from astrocytes in culture. *J Biol Chem* 278:1354–1362.
- Coquelle T, Christensen JK, Banke TG, Madsen U, Schousboe A, Pickering DS (2000) Agonist discrimination between AMPA receptor subtypes. *NeuroReport* 11:2643–2648.
- Cossart R, Tyzio R, Dinocourt C, Esclapez M, Hirsch JC, Ben-Ari Y, Bernard

- C (2001) Presynaptic kainate receptors that enhance the release of GABA on CA1 hippocampal interneurons. *Neuron* 29:497–508.
- Danbolt NC (2001) Glutamate uptake. *Prog Neurobiol* 65:1–105.
- Diana MA, Marty A (2004) Endocannabinoid-mediated short-term plasticity: depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE). *Br J Pharmacol* 142:9–19.
- Duguid IC, Smart TG (2004) Retrograde activation of presynaptic NMDA receptors enhances GABA release at cerebellar interneuron-Purkinje cell synapses. *Nat Neurosci* 7:525–533.
- Engelman HS, MacDermott AB (2004) Presynaptic ionotropic receptors and control of transmitter release. *Nat Rev Neurosci* 5:135–145.
- Esclapez M, Tillakaratne NJ, Kaufman DL, Tobin AJ, Houser CR (1994) Comparative localization of two forms of glutamic acid decarboxylase and their mRNAs in rat brain supports the concept of functional differences between the forms. *J Neurosci* 14:1834–1855.
- Forti L, Pouzat C, Llano I (2000) Action potential-evoked Ca^{2+} signals and calcium channels in axons of developing rat cerebellar interneurons. *J Physiol (Lond)* 527:33–48.
- Grosche J, Kettenmann H, Reichenbach A (2002) Bergmann glial cells form distinct morphological structures to interact with cerebellar neurons. *J Neurosci Res* 68:138–149.
- Hayashi T, Umemori H, Mishina M, Yamamoto T (1999) The AMPA receptor interacts with and signals through the protein tyrosine kinase Lyn. *Nature* 397:72–76.
- Hioki H, Fujiyama F, Taki K, Tomioka R, Furuta T, Tamamaki N, Kaneko T (2003) Differential distribution of vesicular glutamate transporters in the rat cerebellar cortex. *Neuroscience* 117:1–6.
- Huang H, Bordey A (2004) Glial glutamate transporters limit spillover activation of presynaptic NMDA receptors and influence synaptic inhibition of Purkinje neurons. *J Neurosci* 24:5659–5669.
- Iino M, Goto K, Kakegawa W, Okado H, Sudo M, Ishiuchi S, Miwa A, Takayasu Y, Saito I, Tsuzuki K, Ozawa S (2001) Glia-synapse interaction through Ca^{2+} -permeable AMPA receptors in Bergmann glia. *Science* 292:926–929.
- Jörntell H, Ekerot CF (2003) Receptive field plasticity profoundly alters cutaneous parallel fiber synaptic input to cerebellar interneurons *in vivo*. *J Neurosci* 23:9620–9631.
- Keinänen K, Wisden W, Sommer B, Werner P, Herb A, Verdoorn TA, Sakmann B, Seeburg PH (1990) A family of AMPA-selective glutamate receptors. *Science* 249:556–560.
- Kirischuk S, Kirchhoff F, Matyash V, Kettenmann H, Verkhratsky A (1999) Glutamate-triggered calcium signaling in mouse Bergmann glial cells *in situ*: role of inositol-1,4,5-trisphosphate-mediated intracellular calcium release. *Neuroscience* 92:1051–1059.
- Kullmann DM (2001) Presynaptic kainate receptors in the hippocampus: slowly emerging from obscurity. *Neuron* 32:561–564.
- Li P, Wilding TJ, Kim SJ, Calejesan AA, Huettner JE, Zhuo M (1999) Kainate-receptor-mediated sensory synaptic transmission in mammalian spinal cord. *Nature* 397:161–164.
- Lin SC, Huck JHJ, Roberts JDB, Macklin WB, Somogyi P, Bergles DE (2005) Climbing fiber innervation of NG2-expressing glia in the mammalian cerebellum. *Neuron* 46:773–785.
- Liu QS, Xu Q, Arcuino G, Kang J, Nedergaard M (2004) Astrocyte-mediated activation of neuronal kainate receptors. *Proc Natl Acad Sci USA* 101:3172–3177.
- Liu SJ, Cull-Candy SG (2000) Synaptic activity at calcium-permeable AMPA receptors induces a switch in receptor subtype. *Nature* 405:454–458.
- Liu SJ, Cull-Candy SG (2002) Activity-dependent change in AMPA receptor properties in cerebellar stellate cells. *J Neurosci* 22:3881–3889.
- Matsui K, Jahr CE (2003) Ectopic release of synaptic vesicles. *Neuron* 40:1173–1183.
- Matsui K, Jahr CE (2004) Differential control of synaptic and ectopic vesicular release of glutamate. *J Neurosci* 24:8932–8939.
- Matsushita K, Wakamori M, Rhyu IM, Arai T, Oda SI, Mori Y, Imoto K (2002) Bidirectional alterations in cerebellar synaptic transmission of *tottering* and *rolling* Ca^{2+} channel mutant mice. *J Neurosci* 22:4388–4398.
- Min MY, Melyan Z, Kullmann DM (1999) Synaptically released glutamate reduces γ -aminobutyric acid (GABA)ergic inhibition in the hippocampus via kainate receptors. *Proc Natl Acad Sci USA* 96:9932–9937.
- Mitoma H, Konishi S (1999) Monoaminergic long-term facilitation of GABA-mediated inhibitory transmission at cerebellar synapses. *Neuroscience* 88:871–883.
- Nielsen TA, DiGregorio DA, Silver RA (2004) Modulation of glutamate mobility reveals the mechanism underlying slow-rising AMPAR EPSCs and the diffusion coefficient in the synaptic cleft. *Neuron* 42:757–771.
- Obata K, Fukuda T, Konishi S, Ji FY, Mitoma H, Kosaka T (1999) Synaptic localization of the 67,000 mol. wt isoform of glutamate decarboxylase and transmitter function of GABA in the mouse cerebellum lacking the 65,000 mol. wt isoform. *Neuroscience* 93:1475–1482.
- Palay SL, Chan-Palay V (1974) Cerebellar cortex. Cytology and organization. Berlin: Springer.
- Partin KM, Patneau DK, Winters CA, Mayer ML, Buonanno A (1993) Selective modulation of desensitization at AMPA versus kainate receptors by cyclothiazide and concanavalin A. *Neuron* 11:1069–1082.
- Pei XF, Sturgess MA, Valenzuela CF, Maccacchini ML (1999) Allosteric modulators of the AMPA receptor: novel 6-substituted dihydropyridazines. *Bioorg Med Chem Lett* 9:539–542.
- Petralia RS, Wenthold RJ (1992) Light and electron immunocytochemical localization of AMPA-selective glutamate receptors in the rat brain. *J Comp Neurol* 318:329–354.
- Piet R, Vargová L, Syková E, Poulain DA, Oliet SHR (2004) Physiological contribution of the astrocytic environment of neurons to intersynaptic crosstalk. *Proc Natl Acad Sci USA* 101:2151–2155.
- Rodríguez-Moreno A, Herreras O, Lerma J (1997) Kainate receptors pre-synaptically downregulate GABAergic inhibition in the rat hippocampus. *Neuron* 19:893–901.
- Rozas JL, Paternain AV, Lerma J (2003) Noncanonical signaling by ionotropic kainate receptors. *Neuron* 30:543–553.
- Rusakov DA, Kullmann DM (1998) Geometric and viscous components of the tortuosity of the extracellular space in the brain. *Proc Natl Acad Sci USA* 95:8975–8980.
- Rusakov DA, Saitow F, Lehre KP, Konishi S (2005) Modulation of presynaptic Ca^{2+} entry by AMPA receptors at individual GABAergic synapses in the cerebellum. *J Neurosci* 25:4930–4940.
- Saitow F, Satake S, Yamada J, Konishi S (2000) β -Adrenergic receptor-mediated presynaptic facilitation of inhibitory GABAergic transmission at cerebellar interneuron-Purkinje cell synapses. *J Neurophysiol* 84:2016–2025.
- Satake S, Saitow F, Yamada J, Konishi S (2000) Synaptic activation of AMPA receptors inhibits GABA release from cerebellar interneurons. *Nat Neurosci* 3:551–558.
- Satake S, Saitow F, Rusakov D, Konishi S (2004) AMPA receptor-mediated presynaptic inhibition at cerebellar GABAergic synapses: a characterization of molecular mechanisms. *Eur J Neurosci* 19:2464–2474.
- Savtchenko LP, Rusakov DA (2005) Extracellular diffusivity determines contribution of high- versus low-affinity receptors to neural signaling. *NeuroImage* 25:101–111.
- Schenk U, Verderio C, Benfenati F, Matteoli M (2003) Regulated delivery of AMPA receptor subunits to the presynaptic membrane. *EMBO J* 22:558–568.
- Schenk U, Menna E, Kim T, Passafium M, Chang S, De Camilli P, Matteoli M (2005) A novel pathway for presynaptic mitogen-activated kinase activation via AMPA receptors. *J Neurosci* 25:1654–1663.
- Schmitz D, Mellor J, Nicoll RA (2001) Presynaptic kainate receptor mediation of frequency facilitation at hippocampal mossy fiber synapses. *Science* 291:1972–1976.
- Shimamoto K, Lebrun B, Yasuda-Kamatani Y, Sakaitani M, Shigeri Y, Yumoto N, Nakajima T (1998) DL-threo- β -benzyloxyaspartate, a potent blocker of excitatory amino acid transporters. *Mol Pharmacol* 53:195–201.
- Stefanini M, De Martino C, Zamboni L (1967) Fixation of ejaculated spermatozoa for electron microscopy. *Nature* 216:173–174.
- Sugihara I, Wu HS, Shinoda Y (1999) Morphology of single olivocerebellar axons labeled with biotinylated dextran amine in the rat. *J Comp Neurol* 414:131–148.
- Takago H, Nakamura Y, Takahashi T (2005) G protein-dependent presyn-

- aptic inhibition mediated by AMPA receptors at the calyx of Held. *Proc Natl Acad Sci USA* 102:7368–7373.
- Takayasu Y, Iino M, Ozawa S (2004) Roles of glutamate transporters in shaping excitatory synaptic currents in cerebellar Purkinje cells. *Eur J Neurosci* 19:1285–1295.
- Takayasu Y, Iino M, Kakegawa W, Maeno H, Watase K, Wada K, Yanagihara D, Miyazaki T, Komine O, Watanabe M, Tanaka K, Ozawa S (2005) Differential roles of glial and neuronal glutamate transporters in Purkinje cell synapses. *J Neurosci* 25:8788–8793.
- Tamamaki N, Yanagawa Y, Tomioka R, Miyazaki J-I, Obata K, Kaneko T (2003) Green fluorescent protein expression and colocalization with calretinin, parvalbumin, and somatostatin in the GAD67-GFP knock-in mouse. *J Comp Neurol* 467:60–79.
- Tóth K, McBain CJ (1998) Afferent-specific innervation of two distinct AMPA receptor subtypes on single hippocampal interneurons. *Nat Neurosci* 1:572–578.
- Wadiche JI, Jahr CE (2001) Multivesicular release at climbing fiber-Purkinje cell synapses. *Neuron* 32:301–313.
- Wang Y, Small DL, Stanimirovic DB, Morley P, Durkin JP (1997) AMPA receptor-mediated regulation of a G_i-protein in cortical neurons. *Nature* 389:502–504.
- Watanabe M, Fukaya M, Sakimura K, Manabe T, Mishina M, Inoue Y (1998) Selective scarcity of NMDA receptor channel subunits in the stratum lucidum (mossy fibre-recipient layer) of the mouse hippocampal CA3 subfield. *Eur J Neurosci* 10:478–487.
- Yamada K, Fukaya M, Shimizu H, Sakimura K, Watanabe M (2001) NMDA receptor subunits GluR1, GluR3 and GluR5 are enriched at the mossy fibre-granule cell synapse in the adult mouse cerebellum. *Eur J Neurosci* 13:2025–2036.
- Zhang JM, Wang HK, Ye CQ, Ge W, Chen Y, Jiang ZI, Wu CP, Poo MM, Duan S (2003) ATP released by astrocytes mediates glutamatergic activity-dependent heterosynaptic suppression. *Neuron* 40:971–982.