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,1 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 (AMPAR) activation. Although the CF-/AMPAR-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-AMPAR 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/Ca2+-permeable AMPARs, philanthotoxin-433, did not affect the CF-induced inhibition but suppressed AMPAR-mediated currents in Bergmann glia. A low-affinity GluR antagonist, y-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,
which produces powerful excitation in Purkinje cells (PCs), suppresses inhibitory GABAergic transmission between interneurons and PCs after activation of AMPA receptors (AMPARs) (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 protein-dependent pathway, leading to inhibition of presynaptic Ca2+ 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; Castejon 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 neurotransmitter 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-/AMPAR-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-AMPARs on BC axon terminals; and (3) GluR2/3-containing AMPARs are detected at axosomal 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 μm 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 cerebellum 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 CaCl2, 1.0 MgCl2, 21.0 NaHCO3, 0.6 NaH2PO4, and 10.0 glucose (bubbled with 95% O2 and 5% CO2 to maintain pH 7.4 at room temperature). AMPAR currents were recorded in some experiments in a Na+-free, 10 mM Ca2+-ACSF containing the following (in mM): 140 N-methyl-D-glucamine, 10 CaCl2, 10 glucose, and 10 HEPES, pH adjusted to 7.4 by HCl (bubbled with 100% O2) (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, Germany) via patch-clamp electrodes (resistance, 2–5 MΩ) 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 μs) 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 BGs and BG, AMPA (5 μM) was applied by iontophoresis (Mitoma and Konishi, 1999) in the presence of 10 μM cyclothiazide (Tocris Cookson, Bristol, UK) to minimize AMPAR desensitization (Partin et al., 1993). Morphology of BG and BC was examined by intracellular staining with biotin (5 mg/ml; Sigma-Aldrich, St. Louis, MO) during whole-cell recordings. Miniature IPSCs (mIPSCs) were recorded from PCs in ACSF containing 0.5 μM 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-δ-glutamylglycine (γ-DGG), (±)-4-(4-aminoethyl)-1,2-dihydro-1-methyl-2-propylcarbamoyl-6,7-methenylethylenophthalazine (SYM2206), α-chloro-1-cyclopentyl-1,3-dipropylxanthine (DPCPX) were obtained from Tocris Cookson; bromohomoibotenic acid [(±)-Br-HIBO], 1-naphthyl acetyl spermine (NASPM), 8-cyclopentyl-1,3-dimethylxanthine (CPT), and reactive blue-2 (RB-2) were obtained from Sigma-Aldrich; phan-thothoin-433 (PHTX) was obtained from RBI (Natick, MA); and thapsigargin and dextran were obtained from Wako Pure Chemical Industries (Osaka, Japan). threo-β-Benzoxylaspartic 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 titrated with AMPA (0.5 μM) for at least 3 min and then the PC 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 (Savchenko 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 immered 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 μm and mounted on glass slides.

For double-immunofluorescence staining of 65 kDa isofrom 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 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 rodamine-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 rodamine-conjugated goat anti-mouse IgG between anti-GAD65 or anti-synaptophysin antibody and biotinylated goat anti-rabbit IgG and between anti-VLUT2 antibody and rodamine-conjugated goat anti-mouse IgG. After rinsing several times, sections were overslipped 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 μm 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) instead of anti-GAD65. Double-immunofluorescence staining of GAD65 and VGLUT2 were similarly done using biotinylated goat anti-GluR antibody and rodamine-conjugated goat anti-mouse IgG between anti-GAD65 or anti-synaptophysin antibody and biotinylated goat anti-rabbit IgG and between anti-VLUT2 antibody and rodamine-conjugated goat anti-mouse IgG. After rinsing several times, sections were overslipped and observed with a confocal laser scanning microscopy (Bio-Rad, Hemel Hempstead, UK).

**Electron microscopy.** Sections (80 –90 nm) were then obtained from the series of ethanol and acetone. The blocks were infiltrated with resin and stained sections into smaller rectangular blocks that included the molecular layer and the granule cell layer. The blocks were postfix 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 using a Philips EM208 scanning electron microscope (Bio-Rad, Hemel Hempstead, UK).

**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_{50p} ~5 μM) for GluR2 channels, intermediate (EC_{50p} ~80 μM) for GluR2/GluR3 channels, and low (EC_{50p} >200 μM) for GluR3 channels in a *Xenopus* oocyte expression system (Coquelle et al., 2000).

Application of AMPA (0.5 μM) by perfusion has been shown to produce three distinct actions (Satake et al., 2000, 2004): (1) reduction of the amplitude of stimulation-evoked iIPSCs (eIPSCs); (2) increase in the frequency of spontaneously occurring iIPSCs (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 μM) 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 μM AMPA (Fig. 1A,B; see C for a summary). An AMPAR-specific antagonist, SYM2206 (20 μM) (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 <
indicated by a horizontal bar. AMPA (0.5 M) induced an inward current (downward slow deflection) and increases in 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 μM) induced an inward current (downward slow deflection) and increases in spontaneous synaptic responses, whereas Br-HIBO (5 μM) produced only moderate actions on the same BC. Error bars represent SEM.

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 μM, A) or Br-HIBO (5 μM, B). C, Summary of the increases in sIPSC frequency in PCs induced by AMPA (0.5 μM, n = 7) and Br-HIBO (5 μM, 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 μM) induced an inward current (downward slow deflection) and increases in spontaneous synaptic responses, whereas Br-HIBO (5 μM) produced only moderate actions on the same BC. Error bars represent SEM.

0.05) (Fig. 2A–C), which suggests that there might be different subunit compositions between postsynaptic PC-AMPArs and somatodendritic BC-AMPArs, 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. 2D), 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-/AMPAR-mediated disinhibition depends on presynaptic but not BG AMPARs

The above result suggests two possible sites of AMPARs for CF-/AMPAR-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 Ca2+-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 Ca2+ permeability, whereas AMPA-induced responses recorded in BC showed an almost linear I–V relationship and very weak Ca2+ 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 μM), a specific blocker of Ca2+-permeable AMPARs (Tóth and McBain, 1998), strongly suppressed the AMPA-induced currents recorded from BG in both control and Na+-deficient (10 mM Ca2+) 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 Ca2+ permeability.

Based on this striking property of PhTX, we next aimed to determine whether the Ca2+-permeable AMPAR in BG could contribute to the CF-induced inhibition of GABAergic transmission. PhTX (10 μM) 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. 3A–C). Another inhibitor of Ca2+-permeable AMPARs, NASPM (100 μM) (Matsui and Jahr, 2003) also caused no significant effect on the CF-induced suppression of GABA release (Fig. 2C). Conversely, the broad-spectrum AMPAR antagonist SYM2206 (20 μM) markedly suppressed the inhibitory action of CF input on GABAergic transmission (Fig. 3C). Moreover, PhTX did not influence the inhibition of eIPSCs induced not only by the synthetically released CF transmitter but also by exogenous AMPA application (Fig. 3C). Although AMPA increased the frequency of sIPSCs and TTX-insensitive mIPSCs (Satake et al., 2004), these responses were also not affected by PhTX (Fig. 3D). These data rule out Ca2+-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 Ca2+-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 Ca2+ stores (Kirischuk et al., 1999; Coco et al., 2003; Liu et al., 2004). Thus, we examined possible involvements of AMPARs with different subunit compositions between postsynaptic PC-AMPARs 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 Ca2+-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 Ca2+ permeability, whereas AMPA-induced responses recorded in BC showed an almost linear I–V relationship and very weak Ca2+ 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 μM), a specific blocker of Ca2+-permeable AMPARs (Tóth and McBain, 1998), strongly suppressed the AMPA-induced currents recorded from BG in both control and Na+-deficient (10 mM Ca2+) 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 Ca2+ permeability.

Based on this striking property of PhTX, we next aimed to determine whether the Ca2+-permeable AMPAR in BG could contribute to the CF-induced inhibition of GABAergic transmission. PhTX (10 μM) 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. 3A–C). Another inhibitor of Ca2+-permeable AMPARs, NASPM (100 μM) (Matsui and Jahr, 2003) also caused no significant effect on the CF-induced suppression of GABA release (Fig. 2C). Conversely, the broad-spectrum AMPAR antagonist SYM2206 (20 μM) markedly suppressed the inhibitory action of CF input on GABAergic transmission (Fig. 3C). Moreover, PhTX did not influence the inhibition of eIPSCs induced not only by the synthetically released CF transmitter but also by exogenous AMPA application (Fig. 3C). Although AMPA increased the frequency of sIPSCs and TTX-insensitive mIPSCs (Satake et al., 2004), these responses were also not affected by PhTX (Fig. 3D). These data rule out Ca2+-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 Ca2+-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 Ca2+ 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 Ca2+ signaling pathway in the CF-/AMPAR-mediated disinhibition. First, a mixture of purinoceptor antagonists, CPT (0.5 μM) and RB-2 (5 μM) (Zhang et al., 2003) did not affect the CF-induced disinhibition (Fig. 3C). A more selective adenosine A1 antagonist, DPCPX
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 \(\mu M\)) (data not shown). Together, these data argue against the mechanism whereby diffusible messengers, such as ATP/adenosine, endocannabinoids, and cyclooxygenase products (e.g., prostanoids) 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 (Petralia and Wenthold, 1992), antibodies against GluR2 and GluR3 showed intense immunolabeling on PCs 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-spread BG processes that might express GluR1/4. A prominent feature was that double labeling for GluR2/3 subunits and GAD65 (Escalpez 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) (Escalpez 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-
functional interaction between CF and BC terminals. The staining pattern of VGLUT2, 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. 6A,B), reflecting the decrease in the CF transmitter amount after repetitive stimulation. γ-DGG markedly suppressed the CF-induced inhibition of eIPSCs (Fig. 6A,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. 6E). As summarized in Figure 6F, the potency of γ-DGG in suppressing the CF inhibition depended on the frequency of CF stimulation (namely, transmitter concentrations at release sites), being significantly reduced with changing the frequency from 3 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
caused a significant suppression of the CF-induced eIPSC inhibition ($p < 0.05$) (Fig. 6D), 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 $\gamma$-DGG, the extent of this effect did not depend on the frequency of CF stimulation (Fig. 6F).

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 $\mu$M) prolonged the time course of CF-EPSC (Fig. 7A): the decay time constant ($\tau$) of CF-EPSC increased from $7.3 \pm 0.7$ to $13.3 \pm 2.0$ ms ($n = 7$; $p < 0.05$) (Takayasu et al., 2004). Although TBOA (30 $\mu$M) also appeared to prolong PF-EPSC (Fig. 7A), this effect was not significant: the $\tau$ values of PF-EPSC were $7.7 \pm 0.5$ and $9.7 \pm 1.0$ 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. 7B, C), and this effect was markedly suppressed by SYM2206 (Fig. 7C). However, TBOA increased only the magnitude but not the duration of CF-induced IPSC inhibition (Fig. 7D). 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 $\mu$M) caused a profound inhibitory action on stimulation-evoked IPSCs (Fig. 8A); 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. 8C). In contrast to exogenous AMPA (0.5 $\mu$M)-induced inhibition, the TBOA-induced suppression of IPSCs was not associated with changes in the membrane current of PCs (Fig. 8E), 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 $\mu$M) suppressed BC-PC IPSCs and PF-EPSCs to $48.0 \pm 16.2$ ($n = 10$) and $73.8 \pm 14.8\%$ ($n = 6$), respectively ($p < 0.05$, IPSC vs PF-EPSC suppression by TBOA). More importantly, the TBOA (200 $\mu$M)-induced inhibition of eIPSCs was significantly suppressed by the AMPAR antagonist SYM2206 (100 $\mu$M) (Fig.
8B); the eIPSC amplitude decreased by TBOA to 73.7 ± 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 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 BC axon/terminals by escaping from activation of adjacent BG (Matsui and Jahr, 2003, 2004) and other glial cells (Lin et al., 2005).

**Discussion**

It is becoming clear that glutamate mediates not only excitation in the postsynaptic target but also presynaptic regulation of neurotransmitter 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 BC axon/terminals by escaping from activation of adjacent BG (Matsui and Jahr, 2003, 2004) and other glial cells (Lin et al., 2005).
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-AMPArs. 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-/AMPAR-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 presynaptic 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 potential-evoked [Ca^{2+}] rises in BC axonal boutons (Forti et al., 2000).

The presence of GluR2/3-AMPArs in the presynaptic terminal is consonant with the possibility raised recently by Schenk et al. (2003, 2005) that GluR1/2/3-AMPArs 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 diff-
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 CFs, thereby directly inhibiting GABA release at the BC-PC synapse. These observations render strong support for the hypothetical possibility 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. Indeed, the EAAT blocker TBOA augmented the CF-induced inhibition of GABA release or even mimic the inhibitory action of the CF transmitter (Takayasu et al., 2005). 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-AMPARs (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 presynaptic 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


aptic inhibition mediated by AMPA receptors at the calyx of Held. Proc Natl Acad Sci USA 102:7368–7373.