Cellular/Molecular

Metabotropic P2Y Purinoceptor-Mediated Presynaptic and Postsynaptic Enhancement of Cerebellar GABAergic Transmission

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Cerebellar GABAergic inhibitory transmission is under heterosynaptic control mediated by diverse chemical messengers. Here, we investigated roles of metabotropic P2Y purinoceptors (P2YRs) on GABAergic synapses between cerebellar interneurons and Purkinje cells (PCs). Activation of P2Y purinoceptors by two selective agonists, ADP and 2-methylthio-ADP (2MeSADP), elicited two distinct forms of synaptic plasticity of GABAergic transmission in the cerebellar cortex. First, the two agonists induced long-lasting enhancement of stimulation-evoked GABAergic IPSCs as well as $GABA_A$ receptor currents in PCs. This effect was completely abolished by intracellular infusion of the Ca^{2+}-chelating agent BAPTA. Measurements of intracellular Ca^{2+} ($[Ca^{2+}]_i$) dynamics showed that puff application of 2MeSADP produced an increase in $[Ca^{2+}]_i$ of PCs and that this increase persisted in an external Ca^{2+}-deficient medium. These results suggest that P2Y activation postsynaptically elicits long-term enhancement of $GABA_A$ receptor sensitivity of PCs through a G_q-mediated increase in $[Ca^{2+}]_i$. The other action of P2YR agonists on cerebellar GABAergic synapses was that they produced a short-term increase in the frequency and the amplitude of spontaneous $GABA_A$ receptor-mediated IPSCs in PCs in a manner sensitive to a $P2Y_1R$ antagonist, N^6-methyl 2'-deoxyadenosine 3',5'-bisphosphate. This action appeared to be attributable to an excitability increase in presynaptic GABAergic interneurons, because ADP excited all Lugaro cells examined and some of interneurons in the molecular layer. These results suggest that activation of cerebellar P2Y purinoceptors leads to modulation of GABAergic transmission in different spatial and temporal domains, namely short-term and long-term plasticity through presynaptic and postsynaptic mechanisms at interneuron→PC inhibitory synapses in the rat cerebellar cortex.

Key words: cerebellum; Purkinje cell; purinergic; P2Y receptor; GABA; synaptic transmission

Introduction

Neurotransmission at central synapses is under the control of heterosynaptic interactions that use a variety of chemical messengers, including neurotransmitters themselves and other modulators. Among them, ATP and its metabolites have been reported to take part in mediating various forms of chemical signaling through activation of purinoceptors expressed in peripheral tissues and CNS synapses. Purinoceptors have been classified into three main subgroups, adenosine/P1, ionotropic P2X, and metabotropic P2Y receptors (P2XR and P2YR), which differ in their pharmacological profiles, transduction mechanisms, and molecular structures (Ralevic and Burnstock, 1998). These purinoceptor subtypes distribute differentially to neurons and glial cells throughout the mammalian CNS, including the cerebellum

and other brain areas (Evans et al., 1992; Kirischuk et al., 1995; Salter and Hicks, 1995; North and Barnard, 1997; Mateo et al., 1998; North, 2002). Previous studies have shown that activation of adenosine/P1 receptors elicits presynaptic and postsynaptic inhibitory actions on central and peripheral synapses. Furthermore, P2XR and P2YR have been implicated in modulation of neurotransmission at central excitatory glutamatergic synapses (Mendoza-Fernandez et al., 2000; Gomez-Villafuertes et al., 2001; Wirkner et al., 2002). However, relatively little is known about the role played by P2YRs in inhibitory synapses, although their wide distribution in the CNS has been shown by radioligand binding (Simon et al., 1995) and immunohistochemical studies (Moore et al., 2000a,b).

Therefore, this study aimed at examining roles of P2Y purinoceptor activation at inhibitory GABAergic synapses in the cerebellar cortex. Consequently, we find that P2YR agonists elicited short-term and long-lasting enhancement of spontaneous and stimulation-evoked GABAergic transmission between cerebellar interneurons and Purkinje cells (PCs). Furthermore, our findings suggest that the short-term modulation of GABAergic synapses is induced by a presynaptic mechanism of recruiting a population of interneurons, at least in part Lugaro cells, after the P2YR activation and that the P2YR-mediated long-term enhancement is

caused by a postsynaptic mechanism of increasing the $GABA_A$ receptor sensitivity in PCs through G-protein-coupled intracellular Ca^{2+} ($[Ca^{2+}]i$) elevation.

Part of this study has been published previously in abstract form (Saitow et al., 2003).

Materials and Methods

Preparation. Experiments were performed using thin slices cut from the cerebellum of 12- to 18-d-old Wistar rats in accordance with the guidelines of Nippon Medical School (Tokyo, Japan), as described previously (Saitow et al., 2000). In brief, animals of either sex were deeply anesthetized with halothane, and their brains were rapidly removed. Parasagittal slices (250 μm thick) were cut using a vibratome (VT1000S; Leica, Nussloch, Germany) at ~4°C in an Na^+-deficient saline that contained (in mM): 299.2 sucrose, 3.4 KCl, 0.3 $CaCl_2$, 3.0 $MgCl_2$, 10 HEPES, 0.6 NaH_2PO_4, and 10 glucose. The slices were kept for at least 1 h in a humidified interface on a glass-filter chamber supplied with oxygenated artificial CSF (ACSF) that contained (in mM): 138.6 NaCl, 3.4 KCl, 2.5 $CaCl_2$, 1.0 $MaCl_2$, 21.0 $NaHCO_3$, 0.6 NaH_2PO_4, and 10 glucose. The pH of ACSF was maintained at 7.4 by bubbling with 95% O_2–5% CO_2 gas.

Patch-clamp recording. Individual slices were transferred to a recording chamber attached on the stage of a microscope (BX50WI; Olympus, Tokyo, Japan) and continuously superfused with the oxygenated ACSF at a flow rate of 1.5 ml/min and temperature of 25–27°C, and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) was added in the ACSF to isolate $GABA_A$ inhibitory synaptic currents from contaminating ionotropic glutamate receptor-mediated excitatory synaptic responses. Patch electrodes used for whole-cell voltage-clamp recordings from PCs were filled with an internal solution that contained (in mM): 75 CsCl, 75 Cs methanesulfonate, 5.0 KCl, 0.1 K-EGTA, 5.0 Na-HEPES, 3.0 Mg-ATP, and 0.4 Na-GTP, pH 7.4, having resistances of 2–4 MΩ. Whole-cell recordings were also obtained from cerebellar interneurons with electrodes filled with a different internal solution containing (in mM): 150.0 K methanesulfonate, 5.0 KCl, 0.1 K-EGTA, 5.0 Na-HEPES, 3.0 Mg-ATP, and 0.4 Na-GTP, pH 7.4, having resistances of 5–7 MΩ. Neurons were visually identified under Nomarski optics with a water-immersion objective (63×; numerical aperture, 0.90; Olympus). Membrane currents and potentials were recorded with a patch-clamp amplifier (EPC-9; HEKA Elektronik, Lambrecht, Germany), and data were analyzed using PulseFit software (HEKA Elektronik), Kyplot software (Kyence, Tokyo, Japan), and Mini analysis software, version 5 (Synaptosoft, Decatur, GA). Data were also continuously stored during experiments on a videotape recorder after digitizing through a pulse code modulation data recorder for off-line analysis (NF Electronic Instruments, Tokyo, Japan). All signals were filtered at 1 kHz and sampled at 2 kHz, and spontaneous and miniature synaptic currents were analyzed with a threshold of −10 to −40 pA.

Ca^{2+} imaging. Digital fluorescence imaging was performed with a Fluoview confocal microscope system (Olympus) mounted on the microscope. Fluorescence was excited using the 488 nm wavelength of an argon laser, and emissions were detected through a 515 nm barrier filter. For experiments using a cell-permeable Ca^{2+} indicator, Fluo-3 AM (Molecular Probes, Eugene, OR), slices were stained with ACSF containing 5 μM Fluo-3 AM and 0.02% (w/v) pluronic acid F-127 (Sigma, St. Louis, MO) for 30–40 min at room temperature. Before onset of experiments, slices loaded with the indicator were left in normal ACSF for 30 min to allow for hydrolysis of the AM ester. Fluorescence time courses were then recorded in frame scan mode, and images were sampled at 5 s intervals. For experiments using a cell-impermeable Ca^{2+} indicator, Oregon Green BAPTA-1 (OGB-1; Molecular Probes), the indicator was infused into PCs via the recording patch electrode filled with the Cs-base internal solution supplemented with OGB-1 (200 μM). Fluorescence time courses were then recorded in frame scan mode. Images were acquired with a sampling rate of 150 ms per frame while the PC was held at −60 mV. $[Ca^{2+}]_i$ changes are expressed as the ratio of increases in the fluorescence intensity over resting levels ($\Delta F/F_0$). Under the present experimental conditions, there was no significant attenuation of fluores-
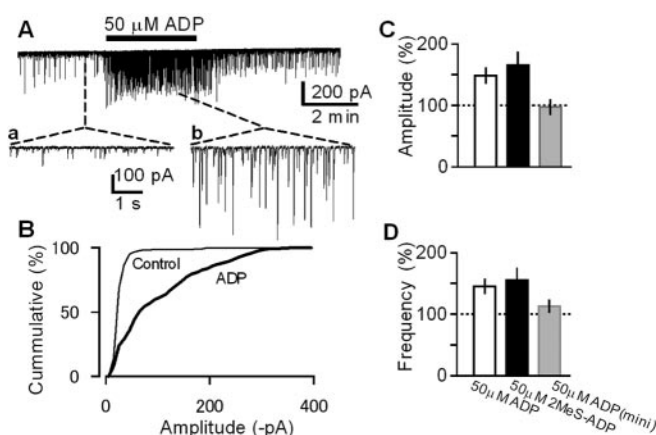
Figure 1. *A*, Short-term effects of a P2YR agonist, ADP, on GABAergic sIPSCs in PCs. ADP (50 μM) was applied by superfusion during the period indicated by a horizontal bar. Bottom traces represent sIPSCs displayed on an expanded time scale before (***Aa***) and during (***Ab***) application of ADP. *B*, Cumulative curves for the amplitude of sIPSCs recorded in a PC before (thin curve; total events, 433; mean, 21.3 ± 1.0 pA) and during (thick curve; total events, 912; mean, 92.7 ± 2.7 pA; $p < 0.001$ for K–S test) ADP application. sIPSCs for 120 s were counted before and under ADP action on the same PC shown in *A*. *C, D*, Effects of two P2YR agonists, ADP and 2MeSADP (50 μM), on the amplitude (*C*) and frequency (*D*) of sIPSCs and miniature IPSCs in PCs. ADP (50 μM) and 2MeSADP (50 μM) were applied by perfusion (open columns for ADP, $n = 8$; filled columns for 2MeSADP, $n = 6$; gray columns for the effects of ADP on miniature IPSCs in the presence of 1 μM TTX, $n = 6$). The P2YR agonists enhanced sIPSCs but did not affect miniature IPSCs. Error bars represent SEM.

cence level (F_0) during the course of imaging unless the cell was stimulated with depolarization pulses.

Chemicals. Research chemicals were obtained from the following sources: ADP and 2-methylthio-ADP (2MeSADP) from Sigma; CNQX, N^6-methyl 2′-deoxyadenosine 3′,5′-bisphosphate (MRS2179), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and 3-N[1-(S)-(3,4-dichlorophenyl)ethyl]amino-2-(S)-hydroxypropyl-P-benzyl-phosphinic acid from Tocris Cookson (Bristol, UK); and tetrodotoxin (TTX) from Sankyo (Tokyo, Japan).

Statistics. Numerical data are given as means ± SEM, where n represents the number of independent experiments. Difference between experimental groups was evaluated using Student's paired or unpaired t test. For the test of differences in cumulative curves, Kolmogorov–Smirnov (K–S) test was used.

Results

Effects of P2YR agonists on cerebellar GABAergic transmission

Using two P2YR agonists, ADP and 2MeSADP, we examined the effect of P2YR activation on cerebellar GABAergic synapses. Application of the P2YR agonists produced two distinguishable actions on $GABA_A$ receptor-mediated transmission onto PCs, as illustrated in Figures 1 and 3. First, ADP transiently increased the amplitude and the frequency of spontaneous IPSCs (sIPSCs) in PCs (Fig. 1*A,C,D*): amplitude increase was 148.4 ± 13.5% of control, and frequency increase was 145.6 ± 12.4% of control ($n = 8$). 2MeSADP also enhanced sIPSCs (see Fig. 3*A,B*): amplitude increase was 165.6 ± 22.1% of baseline, and frequency increase was 155.6 ± 20.0% of baseline ($n = 6$) (Fig. 1*C,D*). Enhancement of sIPSCs by the P2YR agonists showed a fast time course, rapidly attaining a peak effect and reversibly recovering after washout. A conspicuous feature of this enhancement was that ADP recruited a population of sIPSCs with much larger amplitudes (Fig. 1*B*), which suggests that the agonist excites presynaptic GABAergic interneurons and elicits action potential-dependent GABA release. In fact, a transient increase in the sIPSC amplitude after ADP application was completely abolished by 1
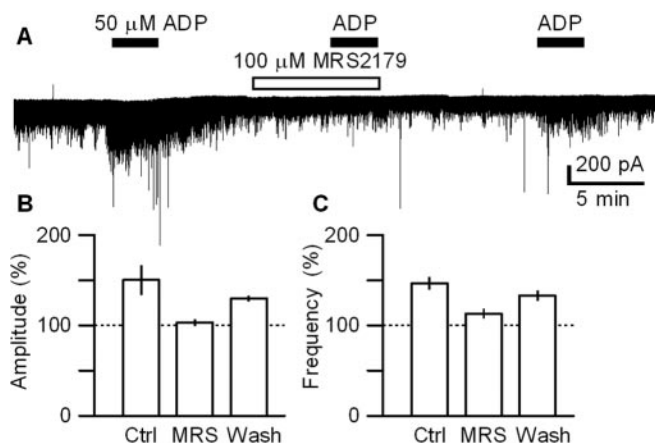
Figure 2. Effects of the P2Y$_1$R antagonist MRS2179 on the ADP-induced short-term enhancement of sIPSCs. **A**, A representative trace showing blockade of the ADP-induced enhancement of sIPSCs after treatment with MRS2179. ADP (50 μM) and MRS2179 (100 μM) were applied during the periods indicated by horizontal filled bars and an open bar, respectively. **B**, **C**, Pooled data for the changes in sIPSC amplitude (**B**) and frequency (**C**) induced by ADP (50 μM) before application [control (Ctrl); $n = 4$], during 100 μM MRS2179 application (MRS; $n = 4$), and 10 min after washing out of the antagonist (Wash; $n = 4$). Error bars represent SEM.

μM TTX (Fig. 1C,D). The ADP-induced transient enhancement of sIPSCs in PCs was markedly suppressed by treatment with the P2Y$_1$R antagonist MRS2179 (Nandanan et al., 2000) (Fig. 2A): in the presence of 100 μM MRS2179, the ADP-induced increases in the amplitude and frequency of sIPSCs were only 103 $\pm$ 3.9 and 111.2 $\pm$ 5.6% of baselines, respectively ($n = 4$) (Fig. 2B,C). The blocking effect of MRS2179 recovered partially 10 min after washing out of the antagonist: the ADP-induced increases in sIPSC amplitude and frequency were 130 $\pm$ 3.5 and 133.1 $\pm$ 5.7%, respectively ($p < 0.05$ vs the MRS2179 treatment; $n = 4$). These pharmacological data indicate that the ADP-induced short-term enhancement of GABA release is mediated through the activation of P2Y purinoceptors.

Second, the P2YR agonist ADP and 2MeSADP also enhanced stimulation-evoked IPSCs (eIPSCs) in PCs (Fig. 3). However, in contrast to the effects on sIPSCs, the onset of enhancement of eIPSCs was gradual, reaching to a peak effect in 20 min and shifting to long-term potentiation (LTP). The extent of eIPSC amplitude increase by ADP and 2MeSADP (determined at 20 min after application) was 148 $\pm$ 31.7 and 134.3 $\pm$ 12.0%, respectively ($n = 8$ for each agonist) (Fig. 3C,D). Although sIPSCs also exhibited a transient increase during the agonist application and a gradual increase in the mean amplitude after the application, the extent of gradual increase in the sIPSCs (open circles) was less than that of eIPSCs (filled circles), as exemplified in Figure 3B, which suggests that spontaneous and evoked GABA release is differentially affected by the purinoceptor activation as a result of heterogeneous localizations of the synaptic sites and/or GABA$_A$ receptor subtypes responsible for sIPSCs and eIPSCs in PCs. ATP also mimicked the ADP-induced short-term enhancement of sIPSCs and LTP of eIPSCs (data not shown).

Postsynaptic mechanisms contribute to P2Y$_1$R-mediated LTP of GABAergic transmission
To determine whether this P2YR agonist-induced LTP of eIPSCs is caused by a presynaptic or postsynaptic mechanism, we compared paired-pulse ratios (PPRs) of eIPSCs (evoked with a 50 ms interval) before and 20 min after the agonist application. The PPR did not show any significant changes (Fig. 3E,F): 0.82 $\pm$
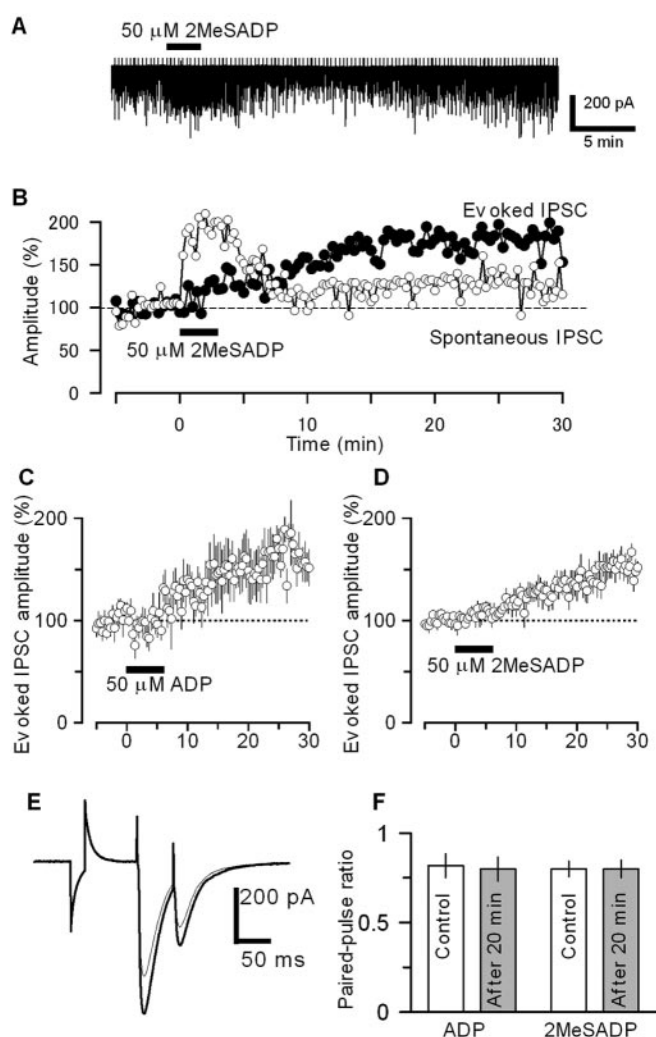

Figure 3. **A**, Time course of 2MeSADP-induced enhancement of sIPSCs in a PC. The P2Y$_1$R agonist 2MeSADP (50 μM) was applied by perfusion during the period indicated by a horizontal bar. The agonist induced two phases of transient and slow-onset increases in sIPSCs and eIPSCs (see also **B**). **B**, Temporal profiles of 2MeSADP-induced increases in the amplitudes of sIPSCs (open circles) and eIPSCs (filled circles). The graph was derived from data in the recording shown in **A**. **C**, **D**, Summary data for the P2YR agonist-induced LTP of eIPSCs in PCs. ADP (**C**; 50 μM; $n = 8$) and 2MeSATP (**D**; 50 μM; $n = 8$) were applied during the period indicated by the horizontal bars. **E**, Example traces of IPSCs evoked by paired-pulse stimulation before (thin trace) and 20 min after (thick line) 50 μM ADP application. **F**, Summary data for the effects of ADP and 2MeSADP on the PPR of eIPSCs in PCs. The PPR was determined before (control; open columns) and 20 min after (gray columns) applying ADP or 2MeSADP. ADP (50 μM; $n = 8$) and 2MeSADP (50 μM; $n = 8$) did not change the PPR during LTP of eIPSCs (gray columns). Error bars represent SEM.

0.07 before and 0.80 $\pm$ 0.07 after application of ADP ($n = 8$; $p > 0.3$) and 0.80 $\pm$ 0.04 before and 0.80 $\pm$ 0.05 after application of 2MeSADP ($n = 8$; $p > 0.8$). It is therefore likely that postsynaptic mechanisms contribute to this LTP. The above results thus indicate that activation of P2Y purinoceptors in the cerebellar cortex can lead to short-term enhancement of sIPSCs with a presynaptic mechanism and LTP of evoked GABAergic transmission with a postsynaptic form at interneuron→PC inhibitory synapses.

To further examine the mechanisms underlying P2YR-mediated modulation of cerebellar GABAergic transmission, we then examined the effect of P2YR agonists on the GABA$_A$ receptor sensitivity of PCs. Both ADP and the P2Y$_1$R-selective agonist 2MeSADP increased the amplitude of GABA$_A$ receptor currents produced in response to brief puff application of exogenous
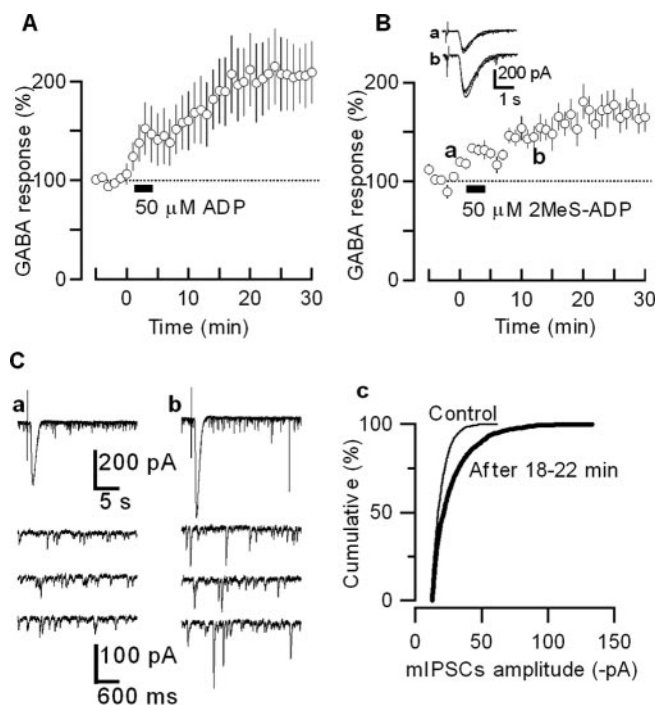
Figure 4. P2YR agonist-induced LTP of GABA$_A$ receptor-mediated currents and mIPSCs in PCs. *A*, *B*, Exogenous GABA (200 μM) was puff applied via a glass micropipette placed in the vicinity of the primary dendritic field of PCs with constant pressure pulses (50 –100 ms), and a 50 μM concentration of either ADP (*A*; n = 10) or 2MeSATP (*B*; n = 12) was applied by perfusion during the period indicated by the horizontal bars. Inset traces represent superimposed successive GABA-induced current responses recorded before (*a*) and 15 min after (*b*) 2MeS-ADP application indicated in *B*. Error bars represent SEM. *C*, mIPSCs were recorded from a PC before (*Ca*) and 20 min after (*Cb*) application of 50 μM ADP. The top trace in *Ca* and *Cb* shows a GABA-induced current response followed by mIPSCs displayed on a slow sweep. Three traces below show consecutive records (3 s) of mIPSCs displayed on an expanded time scale. In *Cc*, cumulative curves for the amplitude distribution of mIPSCs were complied from records during a constant period (5 min) before ADP application (thin curve; total events, 1126; mean, 19.1 ± 0.2 pA) and after 18 –22 min of ADP application (thick curve; total events, 1296; mean, 25.4 ± 0.4 pA; p < 0.001 for K–S test).

GABA from the micropipette placed in the vicinity of PC primary dendrites in the presence of 1 μM TTX (Fig. 4*A*,*B*). The extent of GABA current increase by ADP and 2MeSADP (determined 3 min after agonist applications) was 152.5 ± 26.5% (n = 10) and 132.0 ± 5.6% (n = 12), respectively. These effects were also slowly developing and long lasting, reaching peak levels in 20–30 min: 208 ± 24.5% for ADP (n = 10) and 180 ± 13.6% for 2Me-SADP (n = 12), and there was no significant difference in the magnitudes of enhancement induced by the two agonists (p > 0.7). The time course of the P2YR agonist-induced increase in GABA currents was almost consistent with that of eIPSC enhancement (Fig. 3*C*,*D*). Furthermore, the two agonists increased the mean amplitude of miniature IPSCs (mIPSCs) in PCs (Fig. 4*C*). The P2YR agonist-induced increase in mIPSC amplitude was also gradual and long lasting (Fig. 4*Cc*), being consistent with the changes in eIPSCs and exogenous GABA-induced currents. These results suggest that P2YR activation elicits slow-onset LTP of GABAergic transmission at interneuron→PC synapses through a postsynaptic mechanism.

Intracellular Ca^{2+} increase triggers P2Y$_1$R-mediated LTP at cerebellar GABAergic synapses

It has been shown previously that purinoceptor agonists, such as ATP and ADP, increase the level of cytoplasmic Ca^{2+} in PCs

(Kirischuk et al., 1996) and that the increase in the GABA$_A$ receptor sensitivity of PCs after stimulation of climbing fiber inputs [referred to as "rebound potentiation" (RP)] is associated with a depolarization-induced increase in intracellular Ca^{2+} level (Kano et al., 1992). Therefore, in the experiments illustrated in Figure 5, we sought to determine whether the P2YR-mediated LTP of GABAergic transmission is triggered by intracellular Ca^{2+} elevation in PCs. To monitor [Ca^{2+}]$_i$ dynamics in PCs, we used Fluo-3 AM, a cell-permeable calcium indicator (loaded extracellularly), and Oregon green BAPTA-1, a cell-impermeable indicator (loaded intracellularly via the recording patch pipette). Bath application of 50 μM ADP for 2 min transiently increased [Ca^{2+}]$_i$ in the PC layer when measured with Fluo-3 AM (Fig. 5*A*,*B*); the average increase was 28 ± 2.5% at 10–20 s after application (n = 7). This increase was abolished by pretreatment with a potent P2Y$_1$R antagonist, MRS2179: the average change was 0.2 ± 0.3% at 10–20 s after ADP application (p < 0.001; n = 7). After washing out of MRS2179, the ADP-induced [Ca^{2+}]$_i$ elevation recovered (Fig. 5*C*); the average increase was 14.1 ± 0.3% (p < 0.005; n = 6). The results indicate that activation of P2Y$_1$R is responsible for the ADP-induced [Ca^{2+}]$_i$ elevation in PCs. It is also likely that the P2Y$_1$ purinoceptor subtype mediates not only the ADP-induced short-term enhancement of GABAergic transmission at cerebellar interneuron→PC inhibitory synapses but also purinoceptor-mediated LTP of postsynaptic GABA$_A$ receptor currents in PCs via the ADP-induced [Ca^{2+}]$_i$ elevation.
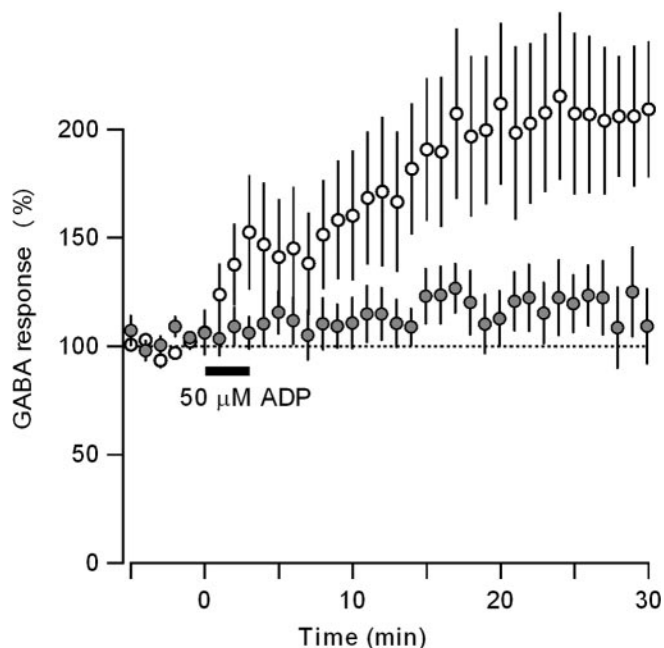
P2Y$_1$R has been classified as a member of G-protein-coupled metabotropic receptors linking to IP$_3$-sensitive Ca^{2+} stores in a variety of cells (Marrelli, 2001; Junankar et al., 2002; Gallagher and Salter, 2003; Hu et al., 2003; Ju et al., 2003). Thus, using combined techniques of electrophysiology and Ca^{2+} imaging, we examined whether purinoceptor activation can increase Ca^{2+} influx or stimulate internal Ca^{2+} stores. To address this issue, we tested the effects of a Ca^{2+}-deficient medium on [Ca^{2+}]$_i$ elevation triggered sequentially by P2YR activation and depolarization in PCs loaded with Oregon Green BAPTA-1 via the recording patch pipette. A depolarizing voltage step (from −60 to 0 mV for 20 ms) induced a sharp increase in [Ca^{2+}]$_i$ in PCs (Fig. 5*D*, Depol.): the average increase was 277 ± 33.8% at the peak in the control solution (n = 5). This response was markedly suppressed in Ca^{2+}-deficient solution: the average increase was only 6.3 ± 1.0% of the control (p < 0.001; n = 6) (Fig. 5*G*). Focal application of 2MeSADP (200 μM, 5000 ms) to dendritic shafts of PCs increased [Ca^{2+}]$_i$ in the dendritic fields (Fig. 5*D*,*E*): the average increase was 52 ± 0.7% at the peak in the control solution (n = 8). However, the P2Y$_1$R agonist-induced Ca^{2+} increase was not significantly altered by the Ca^{2+}-deficient solution: the average increase was 91 ± 14% of the control (p > 0.8; n = 8) (Fig. 5*F*). The effects of the Ca^{2+}-deficient medium were invariably observed when the sequence of application of the agonist and depolarization were alternated (data not shown). The results strongly suggest that intracellular Ca^{2+} mobilization, but not influx, contributes to the P2Y$_1$R-mediated Ca^{2+} elevation, thereby leading to LTP of GABAergic transmission at interneuron→PC inhibitory synapses. Additional support for this idea was provided in the experiment shown in Figure 6, in which we examined the effects of intracellular infusion of a fast Ca^{2+} chelator, BAPTA (20 mM), on the purinoceptor-mediated enhancement of GABA$_A$ receptor currents. BAPTA injection into PCs via the recording pipette virtually abolished LTP of GABA$_A$ receptor-mediated responses at 25 min after ADP application (208 ± 24.5% under control condition and 108.0 ± 12.2% with BAPTA injection; p < 0.001; n = 7).
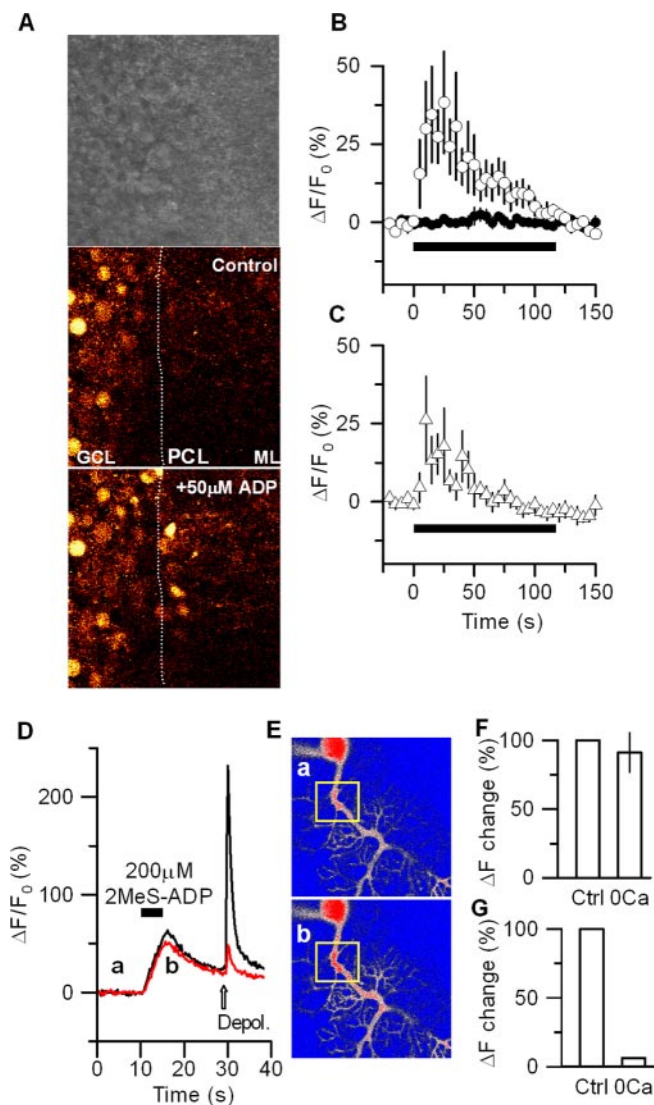
Figure 5. P2YR agonist-induced $[Ca^{2+}]_i$ elevation in PCs. ***A***, Changes in the intracellular Ca^{2+} level were monitored in cerebellar slices loaded with a cell-permeable Ca^{2+} indicator, Fluo-3 AM (see Materials and Methods). The top image shows a field of differential interference contrast view in which fluorescence signals were captured. Middle (control) and bottom (in the presence of 50 μM ADP) images show that application of ADP (50 μM) increased $[Ca^{2+}]_i$ not only in PCs but also in small cells around PCs. ***B***, ***C***, Effects of the P2Y$_1$R antagonist on the ADP-induced intracellular Ca^{2+} increase in PCs. ADP (50 μM) was applied during the period indicated by the horizontal bars in the control ACSF (open circles) and after treatment with 100 μM of the P2Y$_1$R antagonist MRS2179 (filled circles). Blockage of the ADP-induced $[Ca^{2+}]_i$ increase was partially recovered after washing out of MRS2179 in ***C***. ***D***, ***E***, P2YR agonist- and depolarization-induced increases in $[Ca^{2+}]_i$ in single PCs measured using intracellularly loaded Oregon Green BAPTA-1 and effects of a Ca^{2+}-deficient medium. 2MeSADP (200 μM) focally applied to PCs by constant pressure puffs and brief depolarization steps in the same PC elicited an increase in $[Ca^{2+}]_i$ in dendritic fields in control ACSF (black trace), and the depolarization-induced response was markedly suppressed in a nominal Ca^{2+}-free medium, whereas the P2Y$_1$R agonist-induced response remained essentially unaffected (red trace) in ***D***. Images in ***E*** were obtained before (***Ea***) and during (***Eb***) application of ADP in the control ACSF. The yellow rectangular line indicates a region of interest on the dendritic shaft where changes in fluorescence levels were analyzed. ***F***, ***G***, Summary data for the changes in $[Ca^{2+}]_i$ in PC induced by 2MeSADP (***F***) and depolarization of PCs in the control ACSF (Ctrl) and in the Ca^{2+}-deficient medium (0Ca). PCL, Purkinje cell layer. Error bars represent SEM.

Figure 6. Effects of BAPTA infusion on the P2YR agonist-induced LTP of GABA$_A$ receptor currents in PCs. Exogenous GABA was applied to PCs as in Figure 4. ADP (50 μM) was applied by perfusion during the period indicated by a horizontal bar in the control condition (open circles; $n = 10$) and under 20 mM BAPTA injection (filled circles; $n = 9$). BAPTA was injected to individual PCs via the recording patch pipette. Error bars represent SEM.

Identification of presynaptic neurons responsible for P2Y$_1$R-mediated short-term modulation

Because the purinoceptor-mediated short-term enhancement of GABA release is prominent, we sought to determine which

GABAergic neurons are involved in this short-term synaptic plasticity. Possible candidates might be interneurons in the molecular layer (ML), such as basket cells (BCs) and stellate cells (SCs), and Lugaro cells in the granule cell layer (GCL) (Dieudonne and Dumoulin, 2000). To examine the excitability changes after P2Y$_1$R agonist application, current-clamp recordings were made from interneurons in the ML and GCL, to which a constant depolarizing current pulse (+60 pA for 500 ms) was injected, and the number of spikes during the depolarizing pulse was counted before, during, and after 50 μM ADP application (Fig. 7A). Interneurons in the ML (presumably BCs) exhibited different responsiveness to ADP. As shown in Figure 7B, in 4 of 13 ML interneurons, ADP increased the number of spikes during the depolarizing current pulse. However, there was no significant difference in changes in the average number of spikes before and after ADP application (before, 7.2 ± 0.9; during ADP application, 7.3 ± 1.0; after washout, 6.8 ± 1.2; $p > 0.7$; $n = 13$). In 3 of 13 cells, the excitability was decreased after ADP application (control, 8.1 ± 1.3; ADP, 5 ± 1.8; $n = 3$). This inhibitory action of ADP on ML interneurons persisted in the presence of an A$_1$ receptor antagonist, DPCPX (1 μM), which makes it unlikely that a metabolite of ADP, namely adenosine, inhibits these ML interneurons. In contrast, the excitability of Lugaro cells in the GCL was significantly increased by ADP (before, 3.1 ± 0.5; during ADP application, 5.4 ± 0.3; $p < 0.05$; $n = 6$) (Fig. 7C). As reported previously (Dieudonne and Dumoulin, 2000), Lugaro cells could be identified by the shape of fusiform soma, extending processes from both poles in the sagittal plane (Fig. 7D) and almost silent at the resting membrane potential (mean, −60.1 ± 2.9 mV; $n = 13$). Moreover, the number of spikes induced by current injection in Lugaro cells was lower than that in ML interneurons (Fig. 7C).

Because Lugaro cells have been reported to make GABAergic synaptic contacts with PCs (Dean et al., 2003), we attempted to
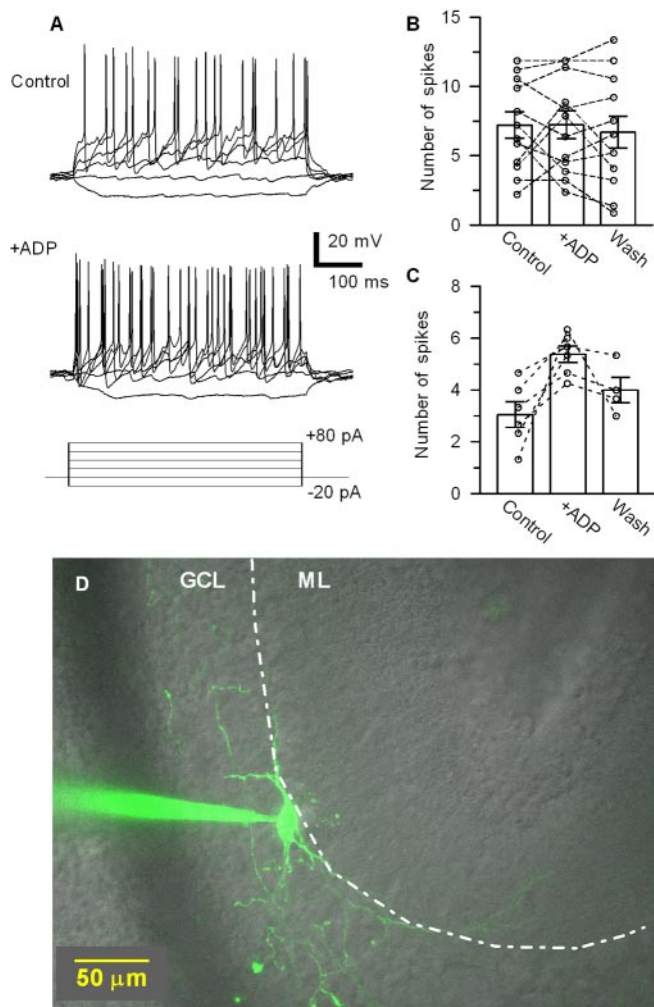
Figure 7. Effects of the P2YR activation on cerebellar GABAergic interneurons in the ML and GCL. **A**, Current-clamp recordings from a single Lugaro cell in the GCL showing membrane potential changes spiking in response to injection of current steps (bottom) in the control ACSF (top; control) and under ADP (50 μM) action (middle; +ADP). **B**, **C**, Summary data for the excitability changes after application of ADP (50 μM) in interneurons (BCs and SCs) in the ML (**B**; $n = 6$) and Lugaro cells (**C**; $n = 13$). The excitability change was estimated by the number of spikes produced in response to constant depolarizing current steps (+60 pA for 500 ms). Each dashed line connecting open circles represents data obtained from the same cells. Error bars represent SEM. **D**, A confocal image of a Lugaro cell stained intracellularly with Alexa Fluor 488 via the recording electrode and superimposed on a transmitted differential interference contrast image of cerebellar slice. The dashed line indicates the PC layer.

perform double recordings from Lugaro cells and PCs. However, we could not detect synaptic connections between them ($n = 13$). Consequently, we then compared the effects of ADP and serotonin (5-HT) on GABAergic synapses on PCs, because 5-HT has been shown to activate Lugaro cells (Dieudonne, 2001), thereby triggering GABAergic synaptic responses in PCs. As shown in Figure 8A, both ADP (50 μM) and 5-HT (10 μM), when sequentially applied to the same PC (at intervals of 5–10 min), elicited GABAergic IPSCs with large amplitudes and increased the frequency of sIPSCs. On average, ADP was more potent than 5-HT in increasing the amplitude and frequency of sIPSCs (Fig. 8 B, C): amplitude increases by ADP and 5-HT were 140.9 $\pm$ 10.3 and 105.5 $\pm$ 3.4% of baseline, respectively ($p < 0.001$; ADP vs 5-HT; $n = 9$), and frequency increases by ADP and 5-HT were 138.0 $\pm$ 7.9 and 111.3 $\pm$ 3.2%, respectively ($p < 0.001$; ADP vs 5-HT; $n = 9$). In addition, the regression analysis showed that there are no
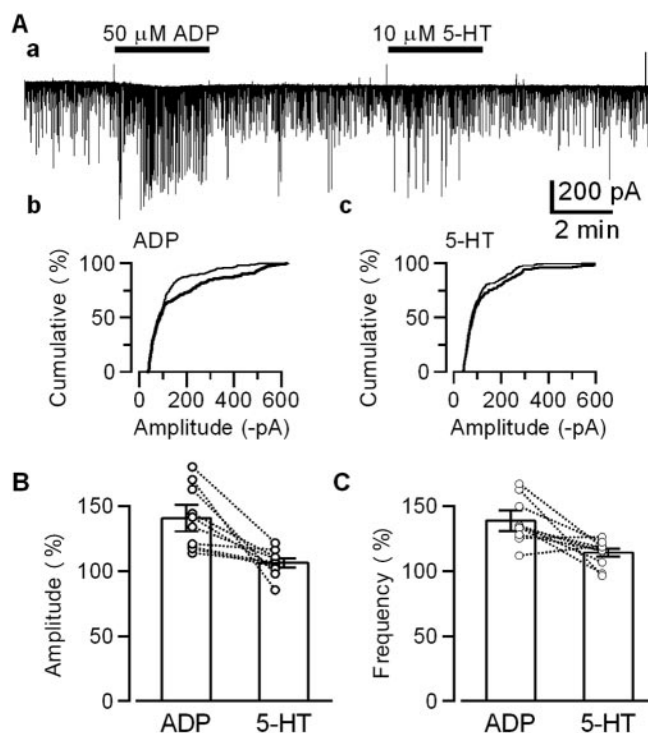

Figure 8. Comparison of the effects of ADP and 5-HT on sIPSCs recorded in PCs. **A**, A representative trace for enhancement of sIPSCs in a single PC after sequential applications of ADP (50 μM) and 5-HT (10 μM). Each agonist was applied by perfusion during the period indicated by a horizontal bar (**Aa**). Cumulative sIPSC amplitude curves were obtained from the record shown in **Aa** before (thin line) and during (thick line) application of ADP (**Ab**) and 5-HT (**Ac**) for a constant period (180 s). In **Ab** and **Ac**, total events and mean amplitudes were 526 and -83.2 ± 3.6 pA before and 754 and -112.1 ± 5.5 pA after ADP, respectively ($p < 0.05$), and 542 and -84.7 ± 3.9 pA before and 638 and -108.7 ± 5.0 pA after 5-HT, respectively ($p < 0.05$). The difference level was assessed by K–S test. **B**, **C**, Summary data for the effects of ADP and 5-HT on sIPSC amplitude (**B**) and frequency (**C**) in PCs. Each column represents the averaged value, and each dotted line connecting open circles indicates data obtained from the same cell. Error bars represent SEM.

significant correlations between the effects of ADP and 5-HT on sIPSC frequency and amplitude: $r = -0.3$ ($n = 9$) for the frequency, and $r = -0.16$ for the amplitude ($n = 9$). Together, the results suggest that P2Y$_1$ purinoceptor-mediated short-term enhancement of GABAergic synaptic activity in PCs is caused by activation of cerebellar interneurons in the ML and GCL and that sIPSCs with large amplitudes induced by P2YR agonists are, at least in part, caused by recruitment of Lugaro cell activation in the GCL. This notion was further supported by the experiments illustrated in Figure 9, in which we examined whether ADP can indeed excite cerebellar interneurons. Both ADP and 5-HT profoundly excited Lugaro cells (Fig. 9A–C). The effects of both agonists on interneurons in the ML were complex: the action-potential firing of some ML interneurons was increased by ADP (Fig. 9D), but these effects were variable among individual ML interneurons (Fig. 9E): 5 of 17 ML interneurons examined (29.4%) were excited by ADP (also see Fig. 7).

Discussion

The finding of this study demonstrates that activation of P2Y$_1$ purinoceptors in the cerebellar cortex elicits two different forms of short-term and long-term plasticity at inhibitory GABAergic synapses between interneurons and PCs. The short-term enhancement of GABAergic synaptic activity in PCs appears to involve a presynaptic mechanism of recruiting a population of Lu-
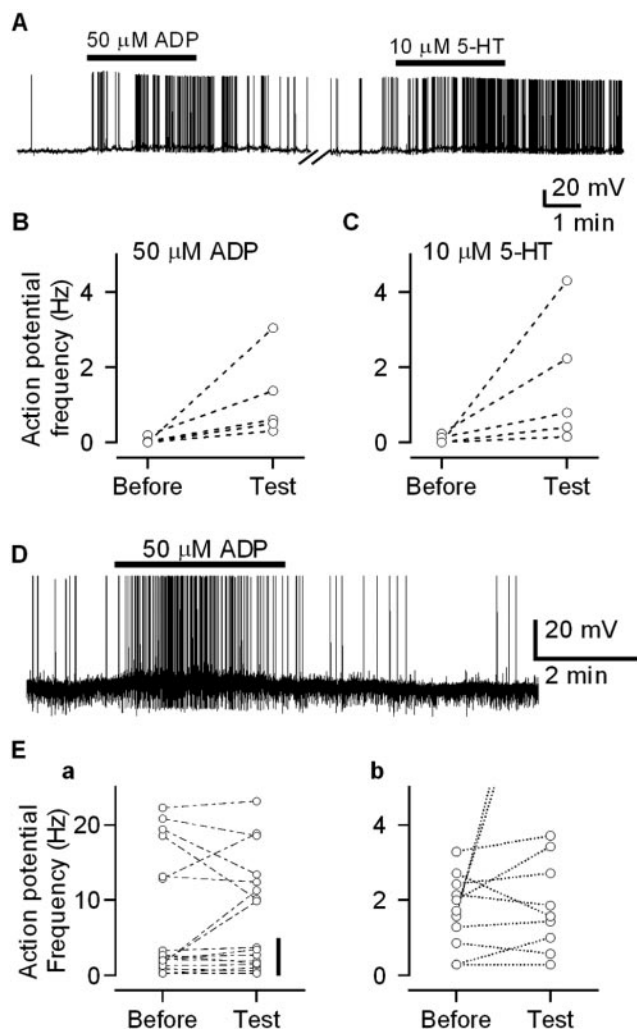
Figure 9. Effects of ADP and 5-HT on action-potential generation in cerebellar GABAergic interneurons in the ML and the GCL. **A**, An example of excitatory actions of ADP and 5-HT on a single Lugaro cell in the GCL recorded under current-clamp mode. Both agonists elicited the firing of action potentials. **B**, **C**, Summary data for changes in the action-potential frequency of Lugaro cells before and after application (Test) of ADP and 5-HT. On average, ADP and 5-HT increased the action-potential frequency from 0.04 ± 0.04 to 1.16 ± 0.5 Hz ($n = 5$) and from 0.07 ± 0.05 to 1.57 ± 0.8 Hz ($n = 5$), respectively. **D**, An example of excitatory action of ADP on an ML interneuron recorded under current-clamp mode. ADP reversibly increased the rate of spontaneous action potential firing (a sign of ML interneurons). **E**, Summary data for changes in the frequency of action potentials of ML interneurons before and after application (Test) of ADP (**Ea**). Part of data indicated in **Ea** by a vertical bar (frequency range, 0 –5 Hz) were expanded in **Eb**. On average, there was no significant difference in action-potential frequency in the absence and presence of ADP (7.4 ± 2.0 and 7.9 ± 1.8 Hz, respectively; $p > 0.7$; $n = 17$).

garo cells in the GCL and GABAergic interneurons in the ML excited after activation of purinoceptors expressed in these interneurons. In contrast, the P2YR-mediated LTP of stimulation-evoked GABAergic transmission onto PCs appears to be induced by a postsynaptic mechanism of increasing the $GABA_A$ receptor sensitivity resulting from intracellular Ca^{2+} elevation caused by G-protein-coupled stimulation of Ca^{2+} stores in PCs. It is therefore strongly suggested that one subtype of prinoceptors, $P2Y_1R$, which is expressed in the cerebellar cortex, plays a distinct role in upregulation of the gain control at cerebellar inhibitory GABAergic synapses in different temporal and spatial domains, thereby inhibiting the excitability level of the cerebellar output PCs.

Presynaptic enhancement of GABA release at cerebellar inhibitory synapses

The metabotropic P2YR-mediated short-term enhancement of inhibitory GABAergic synapses found in the cerebellar cortex is unique, because extracellular nucleotides, such as ATP, have been shown previously to modulate neurotransmitter release at central and peripheral synapses via acting on ionotropic P2X-type purinoceptors (Boehm, 1999; Nakatsuka and Gu, 2001; Khakh et al., 2003; Nakatsuka et al., 2003). The observation in this study is that the $P2Y_1R$ activation increased both frequency and amplitude of sIPSCs in an action potential-dependent manner (Fig. 1), which indicates that $P2Y_1R$ agonists excite presynaptic GABAergic neurons, leading to increased GABAergic synaptic activity in PCs. One possible ionic mechanism that may underlie the P2YR-induced excitation of presynaptic neurons is suppression of potassium channels, because activation of $P2Y_1$-like receptors has been shown recently to inhibit a voltage-dependent K^+ current in *Xenopus* spinal neurons (Brown and Dale, 2002).

Searching for presynaptic neurons responsible for the P2YR-mediated short-term enhancement in PCs revealed that GABAergic Lugaro cells in the GCL and some of the interneurons in the ML are responsive to P2YR agonists, resulting in increases in the excitability (Fig. 7) and direct excitation of these neurons (Fig. 9). The excitability increase in Lugaro cells induced by P2YR agonists was more potent than that induced by 5-HT, which has been shown to elicit GABAergic synaptic activity in PCs through activation of Lugaro cells (Dieudonne, 2001), suggesting that P2Y-type purinoceptors in Lugaro cells also play a pivotal role in modulation of the excitability level of PCs by regulating the activity of GABAergic inputs to the cerebellar output neuron (Dean et al., 2003). Approximately 30% of interneurons in the ML responded to the P2YR agonists (Figs. 7, 9), indicating that they are heterogeneous in terms of the P2Y purinoceptor sensitivity. Because a single PC receives multiple converging GABAergic inputs from 8 to 10 interneurons in the ML (Korbo et al., 1993; Saitow et al., 2000), it seems that individual PCs receive GABAergic inputs from two to three purinoceptor agonist-sensitive interneurons in the ML.

$P2Y_1R$-mediated LTP of GABAergic synapses via a postsynaptic mechanism

Purinoceptor stimulation in the cerebellar cortex was found to induce a distinct form of synaptic plasticity, namely LTP of GABAergic transmission on PCs, through a postsynaptic mechanism. The $P2Y_1R$ agonists elicited a long-lasting increase in stimulation-evoked IPSCs as well as $GABA_A$ receptor sensitivity in PCs (Figs. 3, 4). This $P2Y_1R$-mediated LTP is reminiscent of the phenomenon named rebound potentiation, exhibiting a long-term increase in $GABA_A$ currents in PCs after postsynaptic depolarization (Llano et al., 1991; Kano et al., 1992). Both RP and $P2Y_1R$ LTP were associated with intracellular Ca^{2+} elevation and abolished by intracellular BAPTA injection (Llano et al., 1991; Kano et al., 1992) (Fig. 6). However, there are distinct features between them: (1) the time course of $P2Y_1R$ LTP was much slower than that of RP, and (2) RP depends on Ca^{2+} influx via voltage-gated Ca^{2+} channels (Llano et al., 1991), whereas $P2Y_1R$ LTP persists in Ca^{2+}-deficient medium (Fig. 5). Although both phenomena appear to rely on intracellular Ca^{2+} elevation and subsequent Ca^{2+}-induced Ca^{2+} release and IP_3-induced Ca^{2+} release from intracellular stores (Hashimoto et al., 1996), it is possible that different signaling pathways might be coupled to upregulation of the $GABA_A$ receptor sensitivity in PCs. Although protein phosphorylation of $GABA_A$ receptors mediated by pro-

tein kinase A and calmodulin kinase II is implicated in the RP (Kano et al., 1996; Kawaguchi and Hirano, 2000), it has been reported that GABA$_A$ receptors are phosphorylated by other kinases, such as protein kinase C (Fancsik et al., 2000) and tyrosine kinase (Wan et al., 1997; Dunne et al., 1998). Additional study is therefore needed to determine whether the P2YR-mediated modulation of GABA$_A$ receptor sensitivity also involves protein kinases. It should be noted that the RP in PCs was still able to be induced after depolarization of PCs in the presence of the P2Y$_1$ antagonist MRS2179 (our unpublished observation).

Furthermore, it has been reported recently that activation of metabotropic glutamate receptor 1 in PCs elicits a slow excitatory synaptic response through G$_{q11}$-coupled activation of a transient receptor potential channel 1 (TRPC1) cation channel (Kim et al., 2003) and that metabotropic purinoceptors couple to TRPC7 (Clapham, 2003). However, the P2Y$_1$R activation in PCs did not alter the electrical properties of PCs, such as membrane current and conductance (data not shown), which makes it unlikely that TRPC channels are primary targets of P2Y$_1$ purinoceptors in PCs for the induction of LTP at GABAergic synapses on PCs.

Physiological implications

The purinoceptor-mediated short-term and long-term upregulation of cerebellar inhibitory GABAergic synapses between interneurons and PCs appears to play a critical role in information flow within the cerebellar cortex. Endogenous nucleotides, such as ATP and/or ADP, are potential candidates that mediate the P2YR-activated two forms of synaptic plasticity. ATP has been implicated as an extracellular mediator liberated by astrocytes for chemical communications between glia and neurons (Bezzi and Volterra, 2001; Haydon, 2001). In fact, astroglia display profound Ca^{2+} waves in response to chemical stimuli and thereby elicit massive waves of extracellular ATP (Guthrie et al., 1999). It has been also shown that glutamatergic activity-dependent release of ATP from astrocytes can modulate the strength of neurotransmission in the hippocampus (Zhang et al., 2003). In the cerebellum, Bergmann glia have been shown to receive glutamatergic inputs derived from parallel fibers and climbing fibers (Matsui and Jahr, 2003) and express neurotransmitter receptors, including Ca^{2+}-permeable glutamate receptors and several types of P2Y receptors (Kirischuk et al., 1995; Iino et al., 2001). Therefore, it seems that the Bergmann glial cell is a most likely candidate for the source of nucleotides that take part in the P2YR-mediated short-term and long-term plasticity at cerebellar GABAergic synapses found in this study. This possibility awaits additional examinations.

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