Cellular/Molecular

Group I Metabotropic Glutamate Receptors Inhibit GABA Release at Interneuron-Purkinje Cell Synapses through Endocannabinoid Production

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Actions of endocannabinoids in the cerebellum can be demonstrated following distinct stimulation protocols in Purkinje cells. First, depolarization-induced elevations of intracellular Ca^{2+} lead to the suppression of neurotransmitter release from both inhibitory and excitatory afferents. In another case, postsynaptic group I metabotropic glutamate receptors (mGluRs) trigger a strong inhibition of the glutamatergic inputs from parallel and climbing fibers. Both pathways involve endocannabinoids retrogradely acting on type 1 cannabinoid receptors (CB1Rs) at presynaptic terminals. Here, we show that group I mGluR activation also depresses GABAergic transmission at the synapses between molecular layer interneurons and Purkinje cells. Using paired recordings, we found that application of the group I mGluR agonist (*RS*)-3,5-dihydroxyphenylglycine reduced the evoked IPSCs in Purkinje cells. This effect was independent of postsynaptic Ca^{2+} increases and was completely blocked by a CB1R antagonist.

Experiments performed with the GTP-analogues GDP-βS and GTP-γS provided evidence that endocannabinoids released after G-protein activation can also inhibit GABAergic inputs onto nearby, unstimulated Purkinje cells. Block of the enzymes DAG lipase or phospholipase C reduced the group I mGluR-dependent inhibition, suggesting that 2-arachidonyl glycerol could act as retrograde messenger. Finally, group I mGluR activation by brief bursts of activity of the parallel fibers induced a short-lived depression of spontaneous IPSCs via presynaptic CB1Rs. Our results reveal a mechanism with potential physiological importance, by which glutamatergic synapses induce an endocannabinoid-mediated inhibition of the GABAergic inputs onto Purkinje cells.

Key words: **endocannabinoids; group I metabotropic glutamate receptors; 2-arachidonyl glycerol; 2-AG; GABAergic transmission; paired recordings; cerebellar Purkinje cell**

Introduction

The endocannabinoid system exerts a powerful modulatory action on synaptic transmission in the mammalian brain. Produced on demand after cleavage of membrane precursors (Piomelli, 2003), endocannabinoids can inhibit glutamatergic and GABAergic release in many brain regions (for review, see Alger, 2002; Freund et al., 2003), for example in the cerebellum (Kreitzer and Regehr, 2001a,b; Maejima et al., 2001; Diana et al., 2002; Brown et al., 2003), in the hippocampus (Wilson and Nicoll, 2001; Varma et al., 2001; Ohno-Shosaku et al., 2002b; Kim et al., 2002), and in the cortex (Trettel and Levine, 2003). This modulation is exerted by the activation of type 1 cannabinoid receptors (CB1Rs), the expression of which in the CNS, and in particular in the cerebellum, is primarily concentrated on axonal structures (Tsou et al., 1998; Katona et al., 1999; Egertova and Elphick, 2000; Diana et al., 2002), suggesting a presynaptic site of action for these compounds.

Recent studies have demonstrated that endogenous cannabinoids can function as retrograde messengers. Two main types of protocols triggering endocannabinoid synthesis and release have been characterized. First, endocannabinoids produced in a Ca^{2+}-dependent manner after postsynaptic depolarizations can transiently suppress neurotransmitter release on presynaptic terminals. This form of short-term plasticity has been called depolarization-induced suppression of inhibition (DSI) or depolarization-induced suppression of excitation (DSE), depending on whether the affected afferent inputs are GABAergic or glutamatergic, respectively (Kreitzer and Regehr, 2001a; Ohno-Shosaku et al., 2001, 2002b; Wilson and Nicoll, 2001; Diana et al., 2002; Trettel and Levine, 2003). A second pathway involves G-protein-coupled receptors; activation of group I metabotropic glutamate receptors (mGluRs) and of muscarinic receptors can lead to the release of retrogradely active cannabinoids. In the cerebellum, activation of group I mGluRs by a selective agonist (Levenes et al., 2001; Maejima et al. 2001) or by

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sustained parallel fiber (PF) stimulation (Maejima et al. 2001; Neale et al., 2001; Brown et al., 2003) causes a strong inhibition of EPSCs in Purkinje cells that is blocked by cannabinoid receptor antagonists (Maejima et al. 2001; Brown et al., 2003). A similar linkage between exogenously or synaptically activated group I mGluRs and endocannabinoid production has been found in hippocampal CA1 pyramidal neurons (Varma et al., 2001; Ohno-Shosaku et al., 2002a; Chevaleyre and Castillo, 2003), where also the activation of M_1 and M_3 muscarinic receptor leads to a retrograde modulation of the GABAergic input via endocannabinoid release and CB1R activation (Kim et al., 2002; Ohno-Shosaku et al., 2003). In contrast to DSI–DSE, the pathway activated by G-protein-coupled receptors does not seem to require increases of the intracellular Ca^{2+} concentration (Maejima et al., 2001; Kim et al., 2002).

Using paired recordings, we show here that the release of endocannabinoids by direct activation of group I mGluRs on cerebellar Purkinje cells can also inhibit GABAergic synaptic transmission between molecular layer interneurons and Purkinje cells. Moreover, we report that activation of group I mGluRs by physiological-like stimulation trains of the parallel fibers induces a similar CB1R-mediated depression of spontaneous IPSCs (sIPSCs). The mGluR1-mediated inhibition of GABAergic afferent transmission is thus likely to be a physiologically relevant mechanism for the shaping of the overall synaptic input onto Purkinje cells.

Materials and Methods

Slice preparation. Experiments were performed on slices from the cerebellum of 11- to 18-d-old Sprague Dawley rats. Animals were deeply anesthetized before decapitation. The cerebellar vermis was then isolated and placed in an oxygenated bicarbonate-buffered saline (BBS) at 3–5°C. Parasagittal slices, 180 μm thick, were cut using a VT1000S Vibratome (Leica, Nussloch, Germany), stored for 1 hr at 34°C, and then kept at room temperature for the rest of the experimental day. For electrophysiological recordings, the slices were continuously superfused (1–1.5 ml/min) in the recording chamber with oxygenated BBS. BBS contained the following (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose. The pH was 7.4 after equilibration with 95% O_2 and 5% CO_2.

Electrophysiology. Purkinje and basket–stellate cells were visually identified using an upright Axioskop microscope (Zeiss, Oberkochen, Germany) equipped with differential interference contrast optics and with a water immersion 63×, 0.9 numerical aperture objective. Purkinje cells were voltage clamped in the whole-cell configuration, typically at a holding potential of −60 or −70 mV. Series resistance was compensated for (70–75%) and checked throughout the experiment. For Purkinje cells, we used several intracellular solutions; most of the experiments were performed with an internal solution of the following composition (mM): 109 Cs-gluconate, 20 CsCl, 4.6 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 BAPTA-Cs_4, 4 Na-ATP, 0.4 Na-GTP. In some recordings, the calcium buffer capacity of the internal solution was modified by replacing 10 mM BAPTA with either 1 mM EGTA or 40 mM BAPTA. Changes in osmolarity were compensated for by suitably modifying the content of Cs-gluconate. In all cases, the final osmolarity was 295–300 mOsm. The pH was adjusted to 7.3 with CsOH. For the experiments shown in Figures 4 and 5, GDP-βS or GTP-γS replaced control GTP in the 10 mM BAPTA solution. Molecular layer interneurons were recorded using the perforated-patch technique (Diana et al., 2002). Amphotericin B was dissolved in DMSO (2 mg/30 μl) and added (1/250 dilution) to a solution containing (mM): 135 K-gluconate, 4.6 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES, 1 EGTA, 4 Na-ATP, 0.4 Na-GTP, pH 7.3 with KOH. Presynaptic neurons were voltage clamped at −70 to −80 mV and stimulated with short (3–10 msec) depolarizing pulses (to a holding potential of 0 or 20 mV) sufficient to evoke unclamped action potentials. This stimulation protocol was repeated every 3 sec throughout the entire duration of the experiment. In some experiments, paired stimulations at 30 msec intervals were delivered every 3 sec to presynaptic interneurons to characterize the paired pulse ratio (PPR) of the synaptic connection.

We did not compensate for junction potentials. Recording pipettes were pulled from borosilicate glass capillaries and had a resistance of 3–3.5 MΩ for the Purkinje cells and 10–13 MΩ for interneurons. Stocks of *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM-251) (Tocris Cookson, Bristol, UK), [*S*-(*R**,*R**)]-[3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl)phosphinic acid (CGP 54626) (Tocris Cookson), 7-(hidroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester (CPCCOEt) (Tocris Cookson), 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U73122) (Sigma, St. Louis, MO), and 1,6-bis(cyclohexyloximinocarbonylamino)hexane (RHC 80267) (Calbiochem, La Jolla, CA) were prepared in DMSO. Stocks of 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline (NBQX; Tocris Cookson), D-APV (Tocris Cookson), *S*-3,5-(*RS*)-3,5-dihydroxyphenylglycine (DHPG; Tocris Cookson), TTX (Sigma), bicuculline methochloride (Sigma), 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP; Tocris Cookson), and (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) (Tocris Cookson; a gift from Dr. Carole Levenes, Université Pierre et Marie Curie, Paris, France) were prepared in water. In all experiments, the BBS contained NBQX (2 μM; 10 μM in previous experiments) and D-AP5 (50 μM) to block ionotropic glutamate receptors and CGP 54626 (0.5 or 1 μM) to block $GABA_B$ receptors. All drugs were dissolved directly into the bath BBS solution.

DHPG local application. For the experiments shown in Figure 4*Aa*, the recordings were performed with 0.5 μM TTX, 2 μM NBQX, 25 μM D-APV, 20 μM bicuculline, and 0.5 μM CGP dissolved in the bath BBS. Local applications of DHPG were delivered through a glass pipette filled with the same solution as the extracellular milieu plus 100 μM DHPG. The puffing electrode was placed in the molecular layer at 60–100 μm from the Purkinje cell soma, and short DHPG applications were delivered through an air-pressure controller. Applications were repeated at 5, 15, 30, 45, and 60 min after breaking into the whole-cell configuration.

Extracellular stimulation. In these experiments, we voltage clamped single Purkinje cells with the following intracellular solution (in mM): 135 K-gluconate, 4.6 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES, 1 EGTA, 4 Na-ATP, 0.4 Na-GTP, pH 7.3 with KOH. PFs were stimulated by applying 100 μsec current steps to an extracellular electrode placed in the molecular layer and filled with HEPES-buffered saline. The holding membrane potential was first set to −65 or −70 mV; the position of the extracellular electrode and the stimulus intensity were adjusted to find a reliable PF input, which gave rise to an mGluR1-mediated slow EPSC in the Purkinje cell with a short high-frequency train. Ionotropic glutamatergic transmission was then blocked, and the holding potential was set to −45 or −50 mV, so that sIPSCs appeared as outward currents. For single trials, sIPSCs were recorded during 30 sec windows before and after tetanic stimulation to the parallel fibers consisting of 10 or 30 pulses at 100 Hz. Several (four to seven) tetani were applied before and after application in the bath of either the mGluR1 antagonist CPCCOEt or the CB1R antagonist AM-251. The recordings were performed at both room and more physiological temperatures (32–34°C).

Data acquisition and analysis. Paired recordings were obtained with two patch-clamp amplifiers: a double EPC-9 (Heka Elektronik, Darmstadt, Germany) and an Axopatch 200A (Axon Instruments, Foster City, CA). Data were analyzed using Igor (Wavemetrics, Lake Oswego, OR) and in-house developed routines, as described previously (Diana et al., 2002; Diana and Marty, 2003; Galante and Marty, 2003). Mean control amplitudes of evoked IPSCs (eIPSCs) were calculated by averaging at least 30 amplitude values measured just before DHPG application. The maximal inhibition produced by DHPG was evaluated averaging at least 30 eIPSCs, recorded shortly after the peak of the DHPG-induced inward current. The PPR in control and after DHPG application was given as the percentage ratio between the average amplitude of the second IPSCs and that of the first IPSCs, evaluated over groups of at least 50 eIPSCs for each condition.

For the experiments described in Figure 7, individual sIPSCs were detected and, for each trial, control and test periods were divided into 2

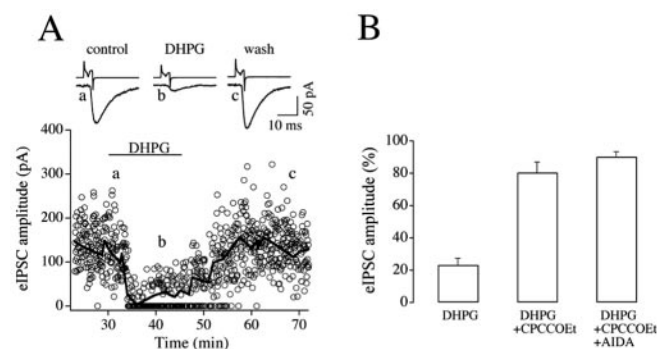

Figure 1. mGluR1 activation inhibits eIPSCs in paired recordings between interneurons and Purkinje cells. *A*, Typical experiment illustrating the inhibitory effect of the group I mGluR agonist DHPG (50 μM) on eIPSC amplitude. Circles correspond to the peak amplitude of single eIPSCs. The amplitude of the evoked IPSCs was 127.1 ± 5.6 pA before DHPG and 10.6 ± 3.5 pA at maximal inhibition. The thick line represents the running average amplitude calculated every 20 eIPSCs; $t = 0$ corresponds to breaking into the whole-cell configuration in the postsynaptic Purkinje cell. Averaged presynaptic (top) and postsynaptic (bottom) traces for sample periods before (*a*), during (*b*), and after (*c*) application of DHPG are shown at the top. *B*, Average eIPSC amplitude after DHPG application from all paired cells tested in control bath solution ($n = 10$) and in the presence of various mGluR1 antagonists. Bath application of the mGluR1 antagonist CPCCOEt (100 μM) greatly reduced the eIPSC depression elicited by DHPG ($n = 6$). Coapplication of CPCCOEt with the other mGluR1 antagonist AIDA (250 μM) decreased the effect of DHPG to a greater extent ($n = 3$). Values are percentage of pre-DHPG control periods.

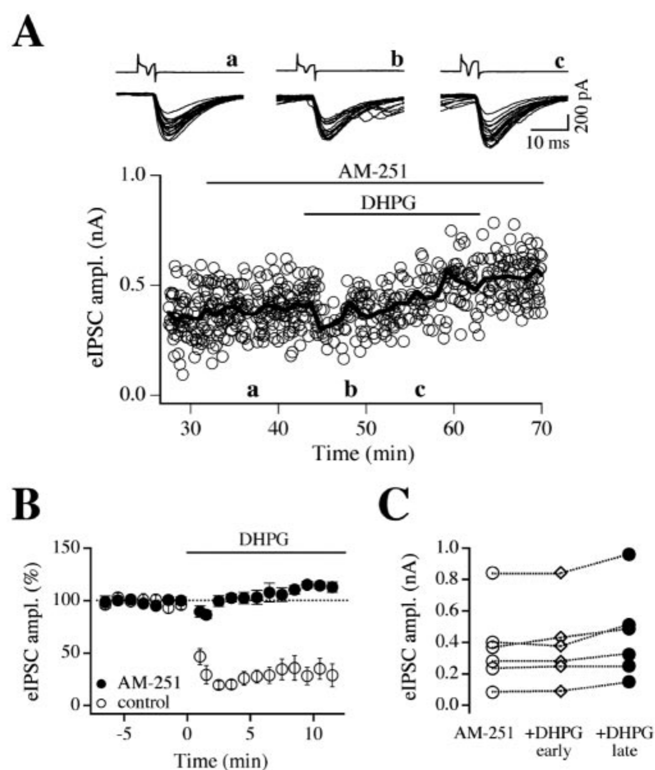

Figure 2. The effect of DHPG on eIPSCs is mediated by endogenous cannabinoids. *A*, Bath application of the CB1R antagonist AM-251 (0.5 μM) prevents the DHPG-induced inhibition of eIPSCs in a typical paired recording. Superimposed individual eIPSCs are shown in the presence of AM-251 (*a*) after 5 min (*b*) and 15 min (*c*) of DHPG application. Note that during the early period of DHPG application, the eIPSCs were not affected by mGluR1 activation (average amplitude was 94.3% of the control). Later, the eIPSC amplitude slowly increased to 127.9% of the control. The eIPSC running average is indicated by the thick line. *B*, Summary time plot from several tested pairs recorded either in the presence (filled circles; $n = 6$) or absence (open circles; $n = 9$) of AM-251; $t = 0$ represents the onset of the mGluR1-mediated inward current in Purkinje cells. Each point represents the average amplitudes of eIPSCs binned into 1 min periods normalized for the average values before DHPG application. *C*, Mean eIPSC amplitudes in the presence of AM-251 (open circles) and at two different times during DHPG application are shown for each tested pair. The early period corresponds to the first 2–4 min in DHPG (diamonds), whereas the late one depicts the period at 10 min after superfusion (filled circles).

sec bins. The amplitudes of the sIPSCs falling into corresponding bins were totaled to give the cumulative amplitude. Bin values were then normalized for the averaged 10–20 sec preceding tetanic stimulation. The corresponding normalized bins from all of the trials in one experimental condition were then averaged, thus giving a normalized time course for the single experiment. Finally, the maximal inhibition of the sIPSC cumulative amplitude was evaluated as the percentage reduction of the averaged bins in the 4–10 sec poststimulation with respect to the control.

Miniature IPSCs (mIPSCs) recorded in 200 nM TTX were detected and binned into 2 sec periods similar to sIPSCs. mIPSC frequencies during control and test periods were calculated as the average values of bins covering at least 2 min. To improve detection, mIPSCs were recorded in Purkinje cells dialysed with KCl replacing K-gluconate in the same solution as that used to record sIPSCs. Statistical comparisons were performed using the Wilcoxon ranked paired test or the Mann–Whitney test. Statistical significance was set at 0.05. Results are given as mean ± SEM.

Results

mGluR1s inhibit GABAergic transmission at the synapses between molecular layer interneurons and Purkinje cells

mGluR1 is the only group I mGluR isoform expressed in cerebellar Purkinje cells (Baude et al., 1993; Negyessy et al., 1997). On Purkinje cell dendrites, these receptors can be located in close apposition not only to glutamatergic terminals but also to GABAergic ones (Baude et al., 1993). Their activation has been shown previously to lead to a presynaptic down-modulation of glutamatergic transmission from afferent parallel and climbing fibers (Levenes et al., 2001; Maejima et al., 2001; Brown et al., 2003). We explored the possibility that postsynaptic mGluR1s could also modulate the GABAergic synapses from molecular layer interneurons.

We recorded from synaptically connected presynaptic basket–stellate cells and Purkinje cells. Vincent and Marty (1996) found an inverse correlation between the synaptic strength of this connection and the depth of location of presynaptic interneurons in the molecular layer. For this reason, our paired recording experiments were primarily performed using presynaptic cells located in the lower two thirds of the molecular layer (basket cells and proximal stellate cells). We used the perforated-patch technique for presynaptic cells to avoid the irreversible run-down of the eIPSCs that is observed in presynaptic whole-cell recordings (Diana and Marty, 2003). eIPSCs were elicited by brief depolarizing pulses provided to the presynaptic interneuron and recorded from postsynaptic voltage-clamped Purkinje cells in the whole-cell configuration (Fig. 1*A*, top).

After a control period indicating a stable synaptic connection, the group I mGluR agonist DHPG (50 μM) was added to the bath solution. DHPG induced three distinct events: (1) a transient inward current, which reflects the opening of cationic channels activated by mGluR1s in Purkinje cells (Batchelor et al., 1994; Tempia et al., 1998; Kim et al., 2003); (2) an increase of frequency of sIPSCs (Llano and Marty, 1995), likely through direct activation of mGluR1s and mGluR5s (Baude et al., 1993; Negyessy et al., 1997) on molecular layer interneurons; and (3) a marked decrease of the eIPSCs (Fig. 1*A*). The inhibition of eIPSCs was maximal 2–4 min after onset of the inward current. No significant correlation between the position of presynaptic cells in the molecular layer and the amount of DHPG-induced inhibition was found; hence, results from basket and stellate cells were

pooled together. On average, the decrease of mean current amplitude was 77.8 ± 4.3% of the control value (range, 53.8–93.4%; $n = 10$) (Fig. 1*B*). Synaptic strength remained strongly depressed throughout the application of DHPG (typically tested for 15–20 min). However, a small recovery developed ~4 min after applying DHPG (Figs. 1*A*, 2*B*, open circles). After 10 min, the depression slightly but significantly recovered to 63.5 ± 9.5% of control ($n = 8$; $p < 0.05$). This corresponds to an eIPSC amplitude of 159.4 ± 29.4% with respect to the maximally depressed value.

After DHPG application, the failure rate of the eIPSCs increased from 3.1 ± 1.3% in control to 55.9 ± 5.3% ($n = 9$; $p < 0.004$). Furthermore, the PPR at 30 msec interstimulus interval significantly augmented from 103.4 ± 5.8 to 154.3 ± 18.9% ($n = 6$; $p < 0.02$). The modification of these parameters strongly supports the hypothesis that DHPG decreases the efficacy of GABAergic transmission via presynaptic mechanisms.

Application of the selective mGluR1 antagonist CPCCOEt (100 μM) greatly reduced the inhibitory effect of DHPG; the amplitude of the eIPSCs was decreased by 19.6 ± 6.5% ($n = 9$; $p < 0.001$) (Fig. 1*B*). In these experiments, mGluR5s were also blocked by coapplication of the potent and selective antagonist MPEP (5 μM) to further evaluate the efficacy of CPCCOEt by monitoring the effect of DHPG on sIPSCs. Because in some cases we noticed that DHPG still elicited an increase of the frequency of sIPSCs, we inferred that mGluR1s were not being completely antagonized by CPCCOEt, and that this was responsible for the persisting inhibition of the eIPSCs by DHPG. Therefore, we performed an additional series of experiments, in which the other selective mGluR1 antagonist AIDA (250 μM) was coapplied with CPCCOEt and MPEP. In this condition, the action of DHPG on the eIPSC amplitude was indeed further reduced (9.8 ± 3.3% inhibition; $n = 3$) (Fig. 1*B*). These results indicate that activation of mGluR1s reduces the strength of GABAergic transmission at the interneuron–Purkinje cell synapse, and that the site of expression of this phenomenon is likely presynaptic.

Endogenous cannabinoids are responsible for the mGluR1-mediated inhibition

Several reports have provided evidence that group I mGluRs can trigger the synthesis and the release of endocannabinoids, which can then inhibit incoming synaptic transmission by activating presynaptic CB1Rs (Maejima et al., 2001; Ohno-Shosaku et al., 2002a; Robbe et al., 2002; Varma et al., 2001; Brown et al., 2003; Chevaleyre and Castillo, 2003). Because CB1R activators powerfully inhibit the GABAergic input onto Purkinje cells [exogenous agonists, Takahashi and Linden (2000), Diana et al. (2002); DSI induction protocols, Diana and Marty (2003)], we tested whether the group I mGluR/endocannabinoids cascade could mediate the inhibitory effect of DHPG on eIPSCs.

Paired recordings between basket–stellate and Purkinje cells were performed in the presence of the specific CB1R antagonist AM-251 (0.5 or 1 μM) (Fig. 2*A*). AM-251 alone led to a small but significant increase of the amplitude of eIPSCs (109.1 ± 3.1% of the baseline; $n = 5$; $p < 0.05$), probably attesting to a basal level of activation of CB1Rs. When DHPG was applied after at least 10 min of AM-251 superfusion, it elicited the typical slow inward current and the increase in sIPSC frequency, which are associated with group I mGluR activation, but did not affect the amplitude of the evoked currents in any of the tested pairs (average, 103.8 ± 3.2%; $n = 6$) (Fig. 2*C*). Thus, the inhibition of eIPSCs by DHPG is mediated by production of endocannabinoids and by activation of CB1Rs.

Interestingly, shortly after group I mGluR activation by DHPG, the amplitude of the eIPSCs progressively increased (Fig. 2). After 10 min in DHPG plus AM-251, the amplitude stabilized to 129.1 ± 10.6% of the control AM-251 conditions (average of $n = 6$ pairs) (Fig. 2*C*). The time course was similar to the recovery observed when DHPG was applied in standard solution (Fig. 2*B*, compare filled and open circles) and quantitatively, the effects were not statistically different ($p > 0.1$). These data suggest that there is a slow, group I mGluR-triggered facilitatory effect, and that this effect is mediated by a pathway independent from the one activated by CB1Rs.

Next, we wondered whether the activation of group I mGluRs and the consequent activation of CB1Rs induced a fully reversible or a long-lasting form of synaptic depression. This is a particularly interesting point, because this same pathway induces long-term depression (LTD) of the GABAergic input onto CA1 pyramidal cells in the hippocampus (Chevaleyre and Castillo, 2003). Thus, we performed an additional series of experiments where AM-251 was added 10–15 min after DHPG superfusion. In AM-251, the eIPSC amplitude fully recovered to 124.8 ± 19.3% of the pre-DHPG period (data not shown; $n = 4$). This value was not statistically different from the one obtained by applying DHPG after CB1R block (see above). Hence, in contrast to the hippocampus, the synaptic inhibition produced during sustained activation of group I mGluRs is entirely mediated by endocannabinoids acting on CB1Rs. No other mechanisms leading to a long-lasting depression of transmission seem to be induced with this protocol in the synapse between basket–stellate and Purkinje cells.

In conclusion thus far, our data provide evidence that activation of group I mGluRs triggers two independent and opposite forms of synaptic modulation: (1) a fast dramatic inhibitory effect attributable to endocannabinoids; and (2) a slower and minor facilitation, which scales up synaptic strength independently from the CB1R-mediated depressing pathway. This latter phenomenon may be related to the tyrosine kinase-dependent mechanism, which was shown previously to increase mIPSC amplitude in Purkinje cells after group I mGluR activation (Boxall, 2000) and thus may be of postsynaptic origin.

Increases of postsynaptic intracellular calcium are not necessary for endocannabinoid production after mGluR1 activation

Postsynaptic calcium rises can play a key role in endocannabinoid signaling, as in the case of DSI–DSE, which are calcium-dependent processes [in the cerebellum, Llano et al. (1991), Kreitzer and Regehr (2001a); in the hippocampus, Pitler and Alger (1992)]. Moreover, some forms of mGluR- and CB1R-dependent LTD are also dependent on elevations of postsynaptic calcium concentration (Choi and Lovinger, 1997; Gerdeman et al., 2002; Robbe et al., 2002). Nevertheless, in some cases, the production of endocannabinoids, which follows G-protein-coupled receptor activation, has been reported to be calcium independent (Maejima et al., 2001; Kim et al., 2002; Chevaleyre and Castillo, 2003).

To investigate whether a rise in postsynaptic calcium is required in the mGluR1-dependent inhibition of GABA release in the cerebellum, we introduced different calcium buffers in postsynaptic Purkinje cells. In all of the experiments presented thus far, an intracellular solution containing 10 mM BAPTA was used. When the calcium buffer was decreased using 1 mM EGTA, DHPG depressed the eIPSC amplitude by 79.5 ± 2.8% with respect to the control ($n = 9$) (Fig. 3*A*), a value very close to that found with 10 mM BAPTA ($p > 0.1$). Similarly, using 40 mM

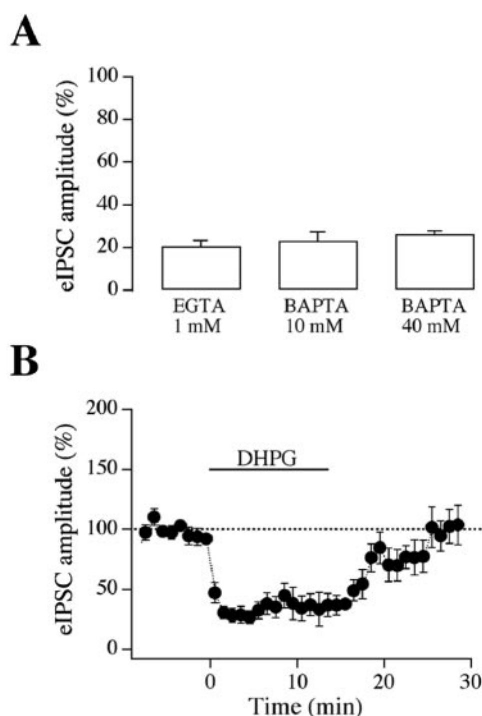

Figure 3. *A*, The mGluR1-induced inhibition is independent from postsynaptic intracellular Ca^{2+} increases. The average eIPSC amplitude during DHPG application is similar when buffering Purkinje cells with 1 mM EGTA ($n = 9$), 10 mM BAPTA ($n = 10$), or 40 mM BAPTA ($n = 4$). The three mean values are not statistically different. *B*, Full-time course of the effect of mGluR1 activation on GABAergic transmission in pairs. Because no significant difference between the various conditions was found, for this plot, we pooled together $n = 2$ pairs with postsynaptic 10 mM BAPTA, $n = 3$ with 40 mM BAPTA, and $n = 2$ with 1 mM EGTA. The graph shows that the effect of DHPG is completely reversible after washout from the bath. $t = 0$ represents the onset of the inward current induced by mGluR1 activation in postsynaptic cells.

BAPTA in the postsynaptic intracellular solution, which is the buffer concentration required to block DSI induction in Purkinje cells (Glitsch et al., 2000), we found a reduction of 73.8 ± 1.5% from the control ($n = 4$; $p > 0.1$) (Fig. 3*A*). These experiments support the view that the production of endocannabinoids via mGluR1s is independent of intracellular calcium increases in Purkinje cells.

The full-time course of the effect of postsynaptic mGluR1 activation on GABAergic transmission could be obtained by pooling together experiments performed with different postsynaptic calcium buffers (Fig. 3*B*, see legend for details). DHPG application reduced synaptic strength by 71.7 ± 5.9% with respect to control but, most importantly, after 15 min of DHPG wash from the bath solution, GABAergic transmission fully recovered to 103.6 ± 16.4% ($n = 8$) of the baseline. Hence, these data confirm that, in our recording conditions, prolonged activations of postsynaptic mGluR1s and of presynaptic CB1Rs do not induce a long-lasting form of depression of GABAergic connections.

Endocannabinoids produced by neighboring cells can contribute to the inhibition of eIPSCs

We have shown that bath-applied DHPG activates mGluR1s, thus inhibiting eIPSCs via CB1Rs. We next tested whether endocannabinoids diffusing from neighboring cells could give a detectable contribution to the mGluR1-mediated depression.

We selectively blocked G-protein activity in the postsynaptic compartment with GDP-βS (2 mM); GDP-βS is a nonhydrolyzable GTP-analog, which inhibits G-protein activity. We monitored the time course of GDP-βS action in the postsynaptic cell as follows: in the presence of a mixture of blockers (see Materials and Methods), we compared the inward currents elicited by local DHPG (100 μM) applications at different times after reaching the whole-cell configuration (Fig. 4*Aa*). The mGluR1-triggered inward current represents a good test for G-protein functionality, because it is known to depend on G-protein activation in Purkinje cells (Tempia et al., 1998; Canepari and Ogden, 2003).

However, the analysis of the effects of GDP-βS was complicated by the fact that, in standard solutions containing GTP, 10 mM BAPTA, and 1 mM Ca^{2+} (estimated free Ca^{2+}, 25 nM), the inward current runs down in time (Fig. 4*Aa*, triangles). This TRPC1-mediated current (Kim et al., 2003) requires high intracellular calcium basal concentrations to be functional (Dzubay and Otis, 2002; Gee et al., 2003). We surmised that the low calcium level could be the reason for the run down, and indeed this was not observed when the calcium concentration in the intracellular solution was increased to 5 mM (estimated free Ca^{2+}, ~220 nM). Therefore, we compared results from control cells loaded with GTP plus 10 mM BAPTA and 5 mM Ca^{2+} (Fig. 4*Aa*, circles) with test cells loaded with GDP-βS plus 10 mM BAPTA and 5 mM Ca^{2+} (Fig. 4*Aa*, squares).

In Purkinje cells loaded with GDP-βS plus 10 mM BAPTA and 5 mM Ca^{2+}, after 30 min in whole cell, the current was reduced on average to 8.5 ± 2.9% ($n = 7$) (Fig. 4*Aa*, squares), whereas in GTP plus 10 mM BAPTA and 5 mM Ca^{2+}, the current amplitude was comparable with that recorded after 5 min in whole cell (99.4 ± 11.5%; $n = 6$) (Fig. 4*Aa*, circles). The difference between control and GDP-βS was significant at all time points ($p < 0.005$). We could therefore conclude that after at least 30 min of GDP-βS diffusion, the inhibition of G-protein activity was almost complete.

On the basis of these results, we performed paired recordings between interneurons and Purkinje cells dialyzed with GDP-βS, and DHPG (50 μM) was added to the bath 35–45 min after entering in the whole-cell configuration (Fig. 4*Ab*). We found that the amount of inhibition produced by mGluR1 activation was extremely variable (Fig. 5*B*). The depression ranged from −7.70 to 70.98% (the two extreme examples are shown in Fig. 4*Ab*). On average, transmission was decreased by DHPG by 36.5 ± 10.2% ($n = 10$). This value was statistically different from that found for control Purkinje cells loaded with GTP (Figs. 1*B*, 5*B*) ($p < 0.005$). These results suggest that endocannabinoids produced in neighboring cells can participate in the inhibition of GABAergic synapses.

We performed an additional series of experiments to support this hypothesis. Pairs of adjacent Purkinje cells were simultaneously whole-cell voltage clamped (Fig. 4*B*); TTX (200 nM) was present in the bath throughout the experiments. One cell was dialysed with a solution containing the nonhydrolyzable G-protein activator GTP-γS (2 mM), whereas mIPSCs were monitored in a nearby Purkinje cell dialysed with a control solution. As Kim et al. (2002) have first shown in hippocampal CA1 cells, inclusion of GTP-γS in the recording pipette can lead to endocannabinoid production and to a subsequent CB1R-dependent inhibition of afferent GABAergic transmission. In our recordings, after a variable time in whole cell, an inward current resembling the mGluR1-mediated cationic conductance developed in the GTP-γS-containing cells. In five of six experiments, mIPSC frequency in the control Purkinje cells decreased shortly after onset of the inward current in the adjacent cells. On average, the frequency decreased to 71.3 ± 9.58% with respect to the control

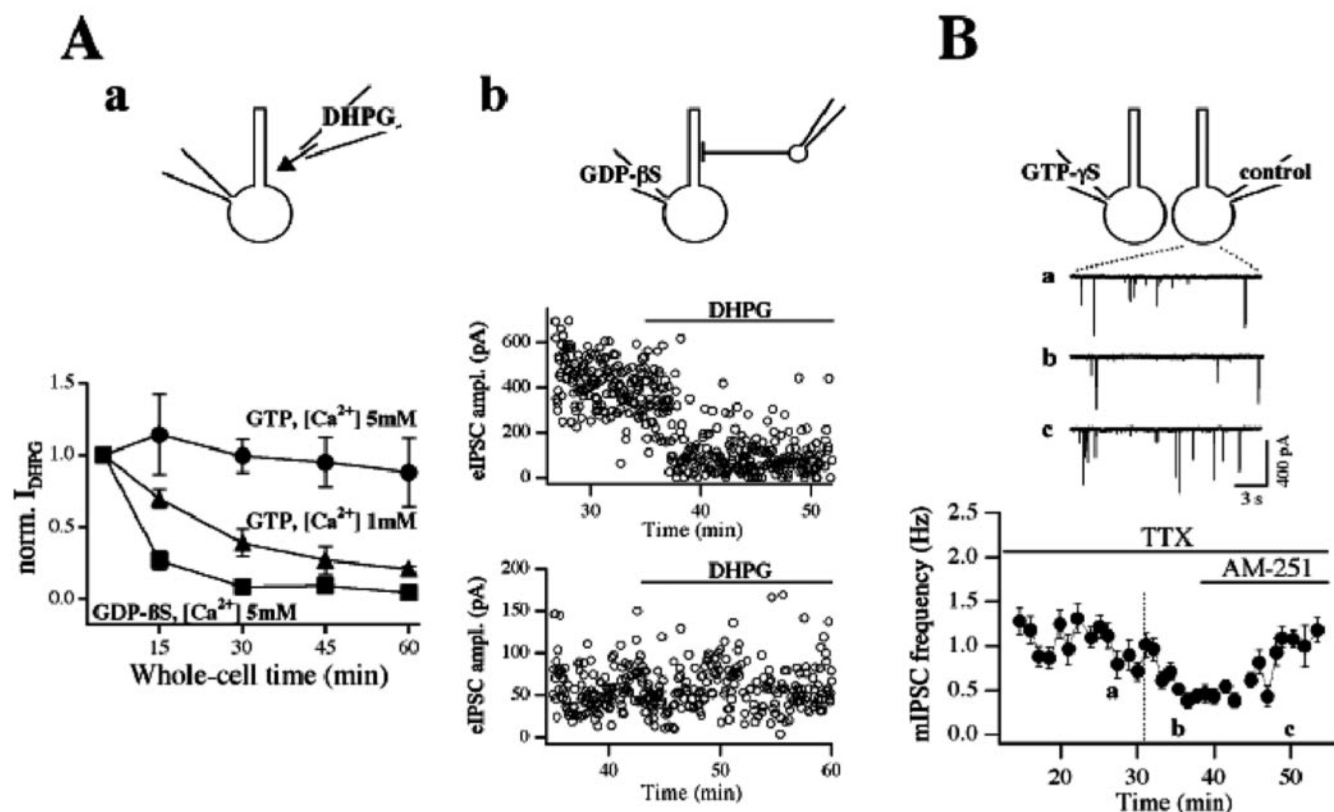

Figure 4. Endocannabinoids diffusing from nearby cells can contribute to the mGluR1-mediated inhibition. The graphics at the top of each section illustrate the experimental configuration corresponding to data collection. *Aa*, Mean amplitude of the currents elicited by DHPG local applications delivered at 5, 15, 30, and 45 min after reaching the whole-cell configuration in Purkinje cells. The internal solution contained 10 mM BAPTA and GTP plus 1 mM Ca^{2+} (triangles; $n = 3$), GTP plus 5 mM Ca^{2+} (circles; $n = 6$), or GDP-βS plus 5 mM Ca^{2+} (squares; $n = 7$). Values are normalized with respect to the currents recorded at 5 min. *Ab*, Two examples of pairs recorded with a postsynaptic intracellular solution containing GDP-βS. The time plots represent the extreme cases found in this set of experiments: a full inhibition of eIPSC amplitude by DHPG (top graph) and a complete block of the effect (bottom graph). In both cases, DHPG was applied at least 35 min after entering the whole-cell configuration, at a time when GDP-βS had blocked G-protein activity, according to the plots in *Aa*. *B*, Simultaneous recording of two adjacent Purkinje cells. mIPSCs were continuously monitored in the cell dialysed with the control solution while the other cell was dialysed with a GTP-γS-containing solution. The mIPSC frequency running average is shown in the bottom plot as a function of whole-cell time in the control cell. In this experiment, the GTP-γS cell was patched at $t = 8$ min. An inward current, similar to that typically activated by mGluR1s, developed in the GTP-γS cell at the time indicated by the vertical dotted line. Shortly afterward, the frequency of mIPSCs decreased from an average 0.89 Hz in control to 0.42 Hz. AM-251 produced a recovery of the frequency to 1.10 Hz. The sample traces were taken during the control period (*a*), during maximal inhibition (*b*), and after AM-251 application (*c*). The distance between the somas of the recorded cells was 25 μm.

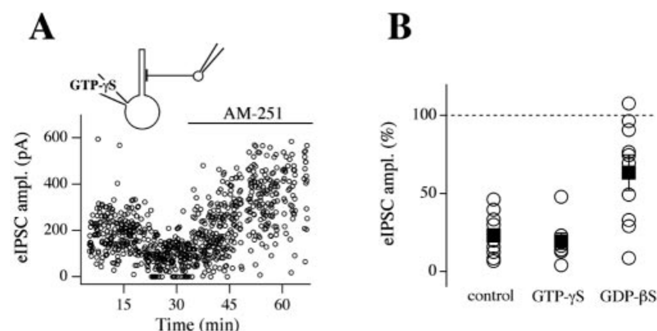

Figure 5. Maximal eIPSC inhibition by G-protein activation in single Purkinje cells. *A*, Loading the postsynaptic Purkinje cell with GTP-γS caused a marked reduction of the amplitude of eIPSCs, which resembled the action of bath-applied DHPG. Addition of AM-251 led to a sustained augmentation of the connection strength. Note the amplitude overshoot after AM-251 application with respect to the period preceding the depression. Average eIPSC amplitudes were 188.6 ± 75.9, 87.5 ± 43.0, and 379.5 ± 158 pA at the start of the recording, during maximal inhibition, and after AM-251 application, respectively. *B*, Summary of the changes induced by DHPG in pairs with postsynaptic internal solutions containing either GTP ($n = 10$) or GDP-βS ($n = 10$) and of the endocannabinoid-dependent inhibition with postsynaptic GTP-γS ($n = 7$). Mean values from all pairs in one group are represented by black squares.

period ($n = 6$). Subsequent application of the CB1R antagonist AM-251 in five of these recordings increased mIPSC frequency to 128.9 ± 18.2% of the control, showing that the depression was attributable to activation of CB1Rs. These experiments indicate that the inhibition of GABAergic transmission can spread from the cell, where the phenomenon is triggered, to nearby unstimulated Purkinje cells.

An evaluation of the maximal CB1R-mediated inhibition after G-protein activation in single Purkinje cells

We then wondered what could be the maximal amount of endocannabinoids, which can be produced after activation of mGluR1s on a single Purkinje cell. Again, we exploited the property of GTP-γS of triggering the production of endocannabinoids through G-protein-related pathways. In paired recordings, we loaded the postsynaptic cell with GTP-γS, thus restricting endocannabinoid production to the recorded Purkinje cell. The complete time course of GTP-γS action onto the eIPSC amplitude could be followed only in a few cases (Fig. 5*A*). Purkinje cells were preferentially patch clamped before presynaptic interneurons and, therefore, in most recordings a paired connection was not available during the first 15–20 min of postsynaptic whole cell. In the cells where the functional pair could be rapidly established, the eIPSC amplitude was found to decrease for the first 20–30

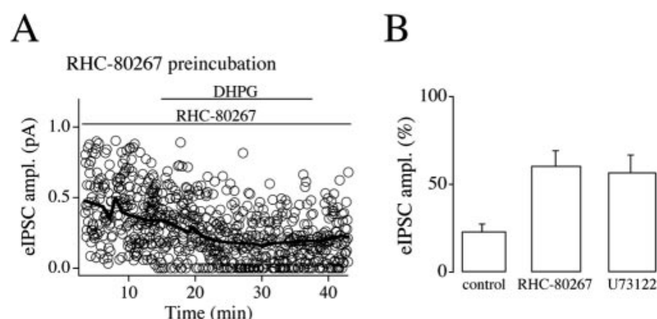

Figure 6. The inhibitory effect of mGluR1 activation on eIPSCs is partly mediated by the endocannabinoid 2-AG. *A*, The inhibitory effect given by DHPG was significantly reduced when slices were incubated in the DAG lipase inhibitor RHC-80267 (100 μM for 1 hr before and 30 μM during recording). In this particular experiment, DHPG reduced the amplitude of the eIPSCs by 56.6%. The black line represents the mean eIPSC amplitude calculated every 20 eIPSCs. *B*, Slice incubation in the presence of RHC-80267 ($n = 7$) or the PLC inhibitor U73122 (5 μM; $n = 5$) partially prevented the effect of DHPG.

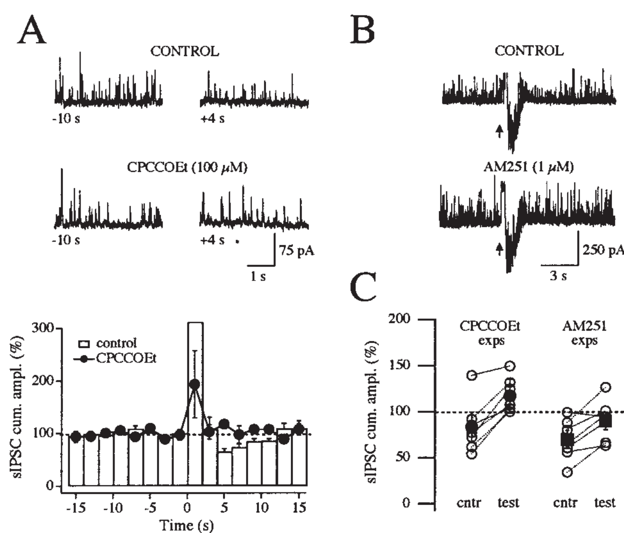

Figure 7. Brief presynaptic bursts of action potentials in the parallel fibers induce a transient depression of sIPSCs in Purkinje cells. *A*, The traces are from an experiment in which the effect on the sIPSC cumulative amplitude of a burst of 10 stimuli at 100 Hz to the parallel fibers was studied. Each sweep is a 4 sec extract taken 10 sec before and 4 sec after a single stimulation trial in control solution (top), and after application of CPCCOEt (bottom). In this recording, the cumulative amplitude of sIPSCs decreased by 46.2% in control and increased by 3.0% in CPCCOEt. The bottom graph shows the average time course of the sIPSC cumulative amplitude for six experiments in control conditions (bars) and after CPCCOEt superfusion (dots). The bin at $t = 0$ (stimulation time) is truncated for clarity. *B*, Two stimulation trials from a single experiment in control conditions (top) and during AM-251 application (bottom) at 34°C in an 18-d-old slice. Note that in the control trace, the slow mGluR1-mediated EPSC produced by 10 stimuli at 100 Hz (arrow) was followed by a decrease in sIPSCs. AM-251 did not affect the slow EPSC but totally blocked the depression of sIPSCs. The slow inward current was totally inhibited by a later application of CPCCOEt (data not shown). For this experiment, the cumulative amplitude of sIPSCs was depressed by 20.2% in control ($n = 4$ trials) and increased by 0.1% in AM-251 ($n = 4$). In the figure, stimulation artifacts were truncated for clarity. *C*, Summary of all experiments (exps); the average cumulative amplitude persisting after the stimulation train before (cntr) and during (test) drug application is shown for the experiments with CPCCOEt (left) and with AM-251 (right). Values from single experiments are connected by a dotted line. Averages from all the cells in a given condition are represented by filled symbols.

min in whole cell before stabilizing (Fig. 5*A*). We then evaluated the contribution attributable to cannabinoid production by adding AM-251 to the bath. In all of the tested pairs, AM-251 invariably led to sustained increase of the connection strength (Fig. 5*B*). On average, the eIPSC amplitude before AM-251 was 19.4 ± 5.2% of the amplitude in AM-251 ($n = 7$). This value was not different from that found for the experiments in which the DHPG-mediated effect was evaluated by superfusing AM-251 after DHPG application in control Purkinje cells (21.6 ± 9.2%; $n = 5$; $p > 0.1$) (see results for the experiments presented in Fig. 2). We conclude that, when maximally stimulated by intracellular GTP-γS, the endocannabinoid synthesis machinery of individual postsynaptic Purkinje cells can produce an eIPSC inhibition comparable with that provided by bath-applied DHPG.

The DHPG-induced decrease in synaptic strength could be partially mediated by the endocannabinoid 2-arachidonyl-glycerol

2-arachidonyl-glycerol (2-AG) is one of the possible endogenous CB1R agonists in the CNS (Stella et al., 1997; Piomelli, 2003). 2-AG is a suitable candidate molecule for the group I mGluR-triggered pathway, because activation of group I mGluRs leads to activation of phospholipase C (PLC) and to production of diacylglycerol (DAG), which is a direct precursor of 2-AG. The conversion of DAG to 2-AG can then be done by a DAG lipase (Bisogno et al., 2003; Piomelli, 2003).

We tested whether the pharmacological agents U73122 and RHC-80267, which respectively inhibit PLC and DAG lipase activity, could affect the mGluR1-mediated inhibition of eIPSCs in pairs. First, slices were incubated for at least 1 hr in U73122 (5 μM) before being moved to the recording chamber, where they were continuously superfused with the same concentration of U7322. In these slices, DHPG still reduced the amplitude of the eIPSCs but only by 43.1 ± 9.8% of the control. The inhibitory effect produced by DHPG was significantly less robust than that found in the absence of U73122 ($n = 5$; $p < 0.005$) (Fig. 6*B*). A similar result was obtained when the DAG lipase inhibitor RHC-80267 was used. The slices were incubated at least 1 hr in RHC-80267 (100 μM) and continuously superfused with 30 μM RHC-80267 in the recording chamber. In these conditions, transmission in pairs was decreased by DHPG by only 39.3 ± 8.6% ($n = 7$; $p < 0.005$); the effect was not statistically different from that found with U73122 ($p > 0.1$). These results suggest that 2-AG, produced by the mGluR1-PLC-DAG lipase pathway, could mediate at least in part the inhibition of GABA release.

Synaptic activation of mGluR1s leads to endocannabinoid production and to the inhibition of GABAergic transmission in Purkinje cells

Bursts of action potentials in parallel fibers can evoke both a heterosynaptic decrease of the climbing fiber input onto Purkinje cells and a synapse-specific self-inhibition (Maejima et al., 2001; Brown et al., 2003); the two phenomena are CB1R mediated. We performed experiments to test whether the production of endocannabinoids after mGluR1 activation by excitatory synaptic inputs can also inhibit the GABAergic input.

We recorded spontaneous IPSCs in Purkinje cells with a K^+-based, low Cl^--containing intracellular solution. Parallel fibers were stimulated via an electrode placed in the molecular layer. We first verified that brief trains of parallel fiber stimulation elicited a mGluR1-activated slow EPSC in Purkinje cells (Batchelor et al., 1994; Tempia et al., 1998) (Fig. 7*B*). Next, fast ionotropic glutamate transmission was blocked, and Purkinje cells were voltage clamped at −45 or −50 mV to record outward GABAergic sIPSCs. After a control period, parallel fibers were extracellularly stimulated 10 times at 100 Hz, and sIPSCs were recorded in the following minute. The stimulation typically elicited a com-

plex, multiphasic response. The frequency of sIPSCs greatly increased in the 4 sec after stimulation and then a depression developed with a slower time course before synaptic transmission returned back to control levels (Fig. 7*A*,*B*).

Experiments were performed at both room (21–23°C) and more physiological (32–34°C) temperatures. The maximal inhibition of the sIPSC cumulative amplitude, calculated as the percentage inhibition between 4 and 10 sec after the end of the stimulation protocol, was 21.0 ± 6.5% ($n = 4$) at room temperature and 18.5 ± 15.8% ($n = 5$) at higher temperatures. These values were not statistically different ($p > 0.10$). Experiments at different temperatures were thus pooled together. Moreover, 10 stimuli at 100 Hz seemed to saturate the inhibitory effect, because 30 pulses at 100 Hz did not give a statistically different maximal inhibition at high temperatures (19.4 ± 14.2%; $n = 7$; $p > 0.10$).

In one series of experiments, the inhibitory effect was studied in either the absence or presence of the mGluR1-specific antagonist CPCCOEt (100 μM). The antagonist completely eliminated the inhibitory effect. Without the blocker, the sIPSC cumulative amplitude during the test period was depressed by 17.0 ± 10.6%, whereas in CPCCOEt, it was facilitated by 16.9 ± 7.1% ($n = 7$; $p < 0.01$) (Fig. 7*A*,*C*). CPCCOEt also significantly, but not completely, reduced the facilitation immediately after the end of the stimulation train (from 367.1 ± 79.4 to 140.1 ± 31.4%; only 10 stimuli experiments were considered; $n = 7$; $p < 0.05$) (Fig. 7*A*). Thus, these protocols are also likely to enhance the activity of molecular layer interneurons through the activation of mGluR1s, which are expressed in these cells (Baude et al., 1993). To test whether the mGluR1-triggered inhibition was mediated by the activation of presynaptic CB1Rs on interneurons, the effect of AM-251 was evaluated with the same experimental protocol (Fig. 7*B*). The inhibition was significantly decreased from 31.6 ± 9.7% in control to 10.3 ± 9.6% in AM-251 ($n = 5$; $p = 0.03$) (Fig. 7*C*).

Finally, we found that this form of inhibition pertains only to parallel fiber activity. Indeed, after brief bursts of activity (five stimuli at 20 Hz) of the climbing fibers at high temperatures, the sIPSC frequency was 103.9 ± 2.56% of the control ($n = 4$). Thus, climbing fibers could not induce a CB1R-mediated inhibition of the GABAergic synapses, although the stimulation protocol was able to activate a slow mGluR1-dependent EPSC as described previously (Dzubay and Otis, 2002). We conclude that brief bursts of action potentials in the parallel fibers, but not in the climbing fibers, heterosynaptically depress GABAergic transmission from molecular layer interneurons onto Purkinje cells via mGluR1 and CB1R activation.

Discussion

In this study, we have shown that in cerebellar slices, the activation of mGluR1s on Purkinje cells can lead to the inhibition of synaptic transmission from GABAergic interneurons. Furthermore, we have characterized this phenomenon in terms of its dependence on the endocannabinoid system. Our work follows the path opened by several recent contributions, which established that the liberation of endocannabinoids by PLC-regulating pathways (both group I mGlu and M_1/M_3 muscarinic receptors) can be an important mechanism leading to the depression of afferent synaptic inputs (Martin and Alger, 1999; Maejima et al., 2001; Neale et al., 2001; Varma et al., 2001; Robbe et al., 2002; Ohno-Shosaku et al., 2002a, 2003; Chevaleyre and Castillo, 2003). Our experiments provide evidence that sustained activation of mGluR1s and CB1Rs triggers a transient modulation of the GABAergic synapses between basket–stellate cells and Purkinje cells. The situation in the cerebellum thus differs from several other brain areas, where repeated activation of the cannabinoid system has been linked to long-lasting forms of synaptic inhibition, both at GABAergic synapses [amygdala, Marsicano et al. (2002); hippocampus, Chevaleyre and Castillo (2003)] and at glutamatergic inputs [striatum, Gerdeman et al. (2002); nucleus accumbens, Robbe et al. (2002); neocortex, Sjöström et al. (2003)].

The heterosynaptic inhibition of GABAergic synaptic inputs by parallel fibers

The basic characterization of the inhibitory effect triggered by mGluR1s was performed by bath applying the agonist DHPG at a saturating concentration. This protocol represented a useful tool to reliably reproduce the phenomenon and study its properties. More physiologically relevant was the finding that synaptic activation of mGluR1s by brief repetitive stimulation of the parallel fibers led to a similar CB1R-dependent inhibition of sIPSCs.

Activation of parallel fibers by extracellular stimulation has been shown previously to induce cannabinoid-mediated inhibition of glutamatergic synapses in Purkinje cells (Maejima et al., 2001; Neale et al., 2001; Brown et al., 2003). In contrast to Maejima et al. (2001), who used strong stimulation protocols of the parallel fibers to obtain a weak inhibitory effect of the climbing fiber input, Brown et al. (2003) reported that more physiological trains of action potentials limited the endocannabinoid-mediated effect to the parallel fibers active during stimulation. Our study expands these studies by reporting that this phenomenon also applies to the GABAergic input onto Purkinje cells. Using similar stimulation protocols, we confirmed the findings of Brown et al. (2003) that brief bursts of action potentials already saturate the amount of physiologically active endocannabinoids produced by parallel fiber activity. Nevertheless, the amount of inhibition with synaptic stimulation was in our case smaller than that obtained with DHPG in pairs. A possible explanation may come from the fact that the extracellular stimulation protocols could activate only a limited subset of the parallel fibers synapsing onto the postsynaptic Purkinje cell, thus modulating via endocannabinoids only few of the active GABAergic synapses. The effect thus may have appeared statistically less robust than in pairs, because the overall population of sIPSCs was sampled for analysis.

We also found that the endocannabinoid-mediated depression of the GABAergic synapses could not be triggered by the climbing fiber input. There are several possible reasons for this finding. First, the physiological range of stimulation frequencies is lower for climbing fibers than for parallel fibers; this could limit the level of activation of mGluR1s and thus the synthesis of endocannabinoids. In addition, other characteristics of the climbing fiber terminals, like their extensive ensheathment by glial structures and the high concentration of glutamate transporters on surrounding membranes, may also reduce the diffusion of glutamate and of endocannabinoids from their sites of release (Palay and Chan-Palay, 1974; Wadiche and Jahr, 2001; Xu-Friedman et al., 2001; Dzubay and Otis, 2002).

On the diffusion of endocannabinoids from nearby cells

The experiments performed with the GTP analogues GDP-βS and GTP-γS have provided valuable information on the mGluR1-dependent depression of the GABAergic input on Purkinje cells. We have shown that the G-protein-coupled pathway of a single postsynaptic cell can produce the same endocannabinoid-driven depression of GABAergic transmission as bath-applied DHPG (Fig. 5). Moreover, a novel finding presented here

is that G-protein-dependent production of endocannabinoids can decrease the GABAergic input onto neighboring unstimulated cells. This finding extends the spatial range of action of this form of modulation and is in contrast to what has been reported previously for the mGluR1-mediated inhibition of the climbing fiber input (Maejima et al., 2001) and for the DSE of the parallel fibers (Kreitzer et al., 2002).

Several mechanisms may underlie the spread of inhibition. First, endocannabinoids could diffuse from their site of release to GABAergic terminals on nearby Purkinje cells and, there, activate CB1Rs. Second, effector molecules produced after a more local activation of CB1Rs may diffuse intracellularly along the presynaptic axon, reach more distant terminals, and inhibit release. One or both of these events may explain the decrease of mIPSC frequency produced by endocannabinoids originating from nearby Purkinje cells, which was presented in Figure 4*C*. Finally, CB1R activation may reduce the firing rate of presynaptic interneurons and the reliability of action potential propagation. This would suppress the synaptic input onto all postsynaptic cells located orthodromically of the site of modulation. In view of these hypotheses, geometry and location of presynaptic structures with respect to sites of endocannabinoid release might determine the amount of spread of inhibition. Morphological reasons may thus explain the marked variability of the inhibitory effect remaining in Purkinje cells loaded with GDP-βS (Figs. 4*Ab*, 5*B*).

Our results were obtained under conditions in which the release of endocannabinoids was presumably persistent over several minutes, both during bath applications of DHPG and during experiments with GTP-γS-loaded Purkinje cells. In this context, it is possible that endocannabinoids may accumulate in the extracellular space and diffuse extensively in the slice. Nevertheless, endocannabinoids can also affect transmission onto distant Purkinje cells when their release is limited to the few seconds after a stimulation protocol. Previous evidence has indeed shown that during DSI, a single Purkinje cell affects GABAergic transmission onto unstimulated Purkinje cells located within 100 μm (Vincent and Marty, 1993). This phenomenon was suggested to be mediated by a reduced efficacy of generation or of propagation of presynaptic action potentials (Vincent and Marty, 1993). Indeed, it was recently ascribed to the fact that endocannabinoids released during DSI upregulate potassium conductances on presynaptic interneurons, thus decreasing their spiking rate (Kreitzer et al., 2002). Recent paired recording results indicate that axonal potassium channels may also decrease either the reliability of action potential propagation into the synaptic terminals or change the spike shape, with possible modifications of the calcium entry per action potential into the terminals (Diana and Marty, 2003). These events may contribute to the mGluR1-triggered inhibition of eIPSC amplitude in pairs and to the spread of inhibition in our paired recordings with postsynaptic GDP-βS. Most importantly, they could be induced by brief periods of activation of the parallel fibers. Parallel fibers might thus modulate the GABAergic input of transversal tracts of the cerebellar cortex by activating mGluR1s on small groups of Purkinje cells.

The identity of the endocannabinoid molecule produced by the mGluR1-triggered pathway

We have shown that blocking DAG lipase or PLC reduces the inhibition of eIPSC amplitude by mGluR1s. Therefore, the G-protein-driven pathway for the synthesis of the endocannabinoid 2-AG involving these enzymes (Di Marzo et al., 1998; Piomelli, 2003) could mediate the phenomenon, and 2-AG could be the retrograde messenger activating CB1Rs on presynaptic GABAergic terminals after brief action potential trains in the parallel fibers. The argument is also supported by the fact that two recently cloned isoforms of DAG lipase are highly expressed in Purkinje cells (Bisogno et al., 2003), which may thus possess the machinery required to produce 2-AG.

The results obtained when blocking PLC and DAG lipase were quantitatively similar. This supports the idea that the partial effect of the inhibitors was not caused by a limited efficacy of the compounds, and that group I mGluRs might simultaneously activate two or more metabolic pathways of endocannabinoid synthesis. It is indeed known that 2-AG can be produced by several independent pathways (Piomelli, 2003), although the evidence in favor of their physiological relevance is still indirect. Intriguingly, by confirming the independence of G-protein-coupled endocannabinoid production from intracellular calcium increases (Maejima et al., 2001; Kim et al., 2002), our data seem to indicate that a separate, Ca^{2+}-dependent, enzymatic cascade is responsible for DSI. A similarly multifaceted situation could characterize hippocampal CA1 cells, in which blocking DAG lipase and PLC completely prevents a calcium-independent form of group I mGluR- and CB1R-mediated LTD of the GABAergic input but leaves DSI totally unaffected (Chevaleyre and Castillo, 2003).

In conclusion, in this study, we characterized a possible mechanism by which glutamatergic transmission onto Purkinje cells can transiently downregulate the concomitant GABAergic input during periods of physiological activity. Future work will hopefully show us which role this form of inhibition may have in the overall processing of the synaptic information conveyed by cerebellar parallel fibers.

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