Developmental Changes in Agonist-Induced Retrograde Signaling at Parallel Fiber–Purkinje Cell Synapses: Role of Calcium-Induced Calcium Release

Francis Crepel and Hervé Daniel
Pharmacologie de la Synapse, Institut de Biochimie et de Biophysique Moléculaire et Cellulaire, Université Paris-Sud, Orsay Cedex, France

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Crepel F, Daniel H. Developmental changes in agonist-induced retrograde signaling at parallel fiber–Purkinje cell synapses: role of calcium-induced calcium release. J Neurophysiol 98: 2550–2565, 2007. First published September 12, 2007; doi:10.1152/jn.00376.2007. In cerebellar Purkinje cells (PCs), activation of postsynaptic mGluR1 receptors inhibits parallel fiber (PF) to PC synaptic transmission by retrograde signaling. However, results were conflicting with respect to whether endocannabinoids or glutamate (Glu) is the retrograde messenger involved. Experiments in cerebellar slices from 10- to 12-day-old rats and mice confirmed that suppression of PF-excitatory postsynaptic currents (EPSCs) by mGluR1 agonists was entirely blocked by cannabimimetic receptor antagonists at this very early developmental stage. In contrast, suppression of PF-EPSCs by mGluR1 agonists was only partly blocked by cannabimimetic receptor antagonists in 18- to 22-day-old rats, and the remaining suppression was accompanied by an increase in paired-pulse facilitation. This endocannabinoid-independent suppression of PF-EPSCs was potentiated by the Glu uptake inhibitor d-threo-benzoyloxyaspartate (t-TBOA) and blocked by the desensitizing kainate (KA) receptors agonist AMPH 3081, by nonsaturating concentrations of 6-cyano-7-nitroquinoline-2-3-dione (CNOX) but not by GYKI 52466 hydrochloride (GYKJ) and by dialyzing PCs with guanosine 5'-[β-thio]-diphosphate (GDP-βS). An endocannabinoid-independent suppression of PF-EPSCs was also present in nearly mature wild-type mice but was absent in GluR6−/− mice. The endocannabinoid-independent suppression of PF-EPSCs induced by mGluR1 agonists and the KA-dependent component of depolarization-induced suppression of excitation (DSE) were blocked by ryanodine acting at a presynaptic level. We conclude that retrograde release of Glu by PCs participates in mGluR1 agonist-induced suppression of PF-EPSCs at nearly mature PF-PC synapses and that Glu operates through activation of presynaptic KA receptors located on PFs and prolonged release of calcium from presynaptic internal calcium stores.

INTRODUCTION

In cerebellar Purkinje cells (PCs), activation of mGluR1 postsynaptic metabotropic glutamate receptors by selective agonists (Galante and Diana 2004; Levenes et al. 2001; Maejima et al. 2002) or by sustained parallel fiber (PF) stimulation (Brown et al. 2003; Maejima et al. 2001; Neale et al. 2001) inhibits both excitatory and inhibitory inputs to these neurons by retrograde signaling (Brown et al. 2003; Galante and Diana 2004; Levenes et al. 2001; Maejima et al. 2001; Marcaggi and Attwell 2005). Endocannabinoids have been favored as the retrograde messenger involved in DSE (Brenowitz and Regehr 2003; Kreitzer and Regehr 2001b; Safo and Regehr 2005). However, the study by Levenes et al. (2001) contrasts with these results, suggesting instead that retrograde release of glutamate (Glu) by PCs was responsible for the observed agonist-dependent suppression of PF-excitatory postsynaptic currents (EPSCs), through activation of presynaptic ionotropic Glu receptors borne by PFs. Because most studies on the role of endocannabinoids in retrograde signaling at PF-PC synapses have been performed in juvenile rats and mice (but see Safo and Regehr 2005), whereas the study by Levenes et al. (2001) was performed in nearly mature rats, the apparent discrepancy may be related to developmental differences. Indeed, we now know that DSE at PF-PC synapses is entirely mediated through retrograde release of endocannabinoids in juvenile rodents, whereas it also involves retrograde release of Glu in nearly mature animals (Crepel 2007).

Therefore these experiments were designed to determine whether such distinct mechanisms are also involved in suppression of PF-EPSCs by activation of postsynaptic mGluR1 in juvenile and nearly mature rats and mice. Because presynaptic kainate (KA) receptors are involved in DSE in nearly mature PF-PC synapses (Crepel 2007), emphasis was made on a possible role of these receptors in agonist-induced suppression of PF-EPSCs in nearly mature PF-PC synapses, as well as on a possible participation of presynaptic calcium-induced calcium release in this process.

METHODS

Experimental procedures complied with guidelines of the French Animal Care Committee. They were performed on juvenile (10–12 days old) and on nearly mature (18–22 days old) male rats (Sprague-Dawley). Additional experiments were also performed on 22- to 24-day-old C56BL/6 and GluR6−/− (on a hybrid 129Sv × C57BL/6 background) mice, as well as on juvenile (10–12 days old) C56BL/6 mice. In all cases, animals were stunned before decapitation, and parasagittal slices, 250 μm thick, were cut in ice-cold saline solution from the cerebellar vermis with a vibroslicer. Slices were incubated at room temperature in saline solution equilibrated with 95% O2–5% CO2 for ≥1 h. The recording chamber was perfused at a rate of 2 ml/min with oxygenated saline solution containing (in mM) 124 NaCl, 3 KCl, 24 NaHCO3, 1.15 KH2PO4, 1.15 MgSO4, 2 CaCl2, 10 glucose,

Address for reprint requests and other correspondence: F. Crepel, Pharmacologie de la Synapse, IBBMC, Bât. 430, Univ. Paris-Sud, 91405 Orsay Cedex, France (E-mail: francis.crepel@u-psud.fr).

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and the GABA_A antagonist bicuculline methochloride (10 μM, Sigma Aldrich, St. Quentin Fallavier, France), osmolarity 320 mOsm, final pH 7.35 at 27–28°C except when otherwise specified. PCs were directly visualized with Nomarski optics through a 40× water-immersion objective of an upright microscope (Zeiss).

Drugs were added to the superfusate. (S)-3,5-dihydroxyphenylglycine (DHPG), domoate, t-threo-β-benzylxospartate (t-TBOA), 6-cyano-7-nitroquinoxaline-2-3-dione (CNQX), GYKI 52646 hydrochloride (GYK1), t-α-amino-5-phosphopentanoic acid (t-APV), (2S,4R)-4-methylglutamate acid (SYM 2081), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), CGP55845-A-(piperidin-1-yl)-5-(4-iodophenyl)-1(2,4dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchased from Tocris (Illkirch, France). The CB1 cannabinoid receptor antagonist SR141716-A [N-(piperidin-1-yl)-5-(4chlorophenyl)-(4-methyl-1H-pyrazole-3-carboxamide (AM-251), and ryanodine were purchase...
Duvoisin 1995; Schoepp et al. 1994), induced a large and significant ($P < 0.001$) decrease in the mean amplitude of PF-EPSCs to 54.17 ± 5.12% of control ($n = 6$; Fig. 1, A and B). This suppression was accompanied by a large and significant ($P < 0.002$) increase in PPF, from 1.35 ± 0.06% in control to 1.86 ± 0.19% at the peak of the DHPG effect. This is in keeping with a presynaptic action of DHPG at PF-PC synapses through retrograde signaling in juvenile mice (Maejima et al. 2001). PF-EPSCs recovered their initial amplitude within <2 min of DHPG application and thereafter were potentiated for several minutes (123.48 ± 8.64% of control on average; Fig. 1, A and B). Very similar results were obtained in 10- to 12-day-old rats because bath application of 100 µM DHPG also induced a reversible decrease in the mean amplitude of PF-EPSCs to 68.22 ± 4.37% of control that was also followed by a transient potentiation of PF-EPSCs ($n = 6$; Fig. 1C). However, this potentiation was shorter than in 10- to 12-day-old mice and was later followed by a transient, albeit not significant, depression of PF-EPSCs that amounted to 8.98 ± 8.95% on average (Fig. 1C). In both mice and rats, the transient potentiation that followed the initial suppression of PF-EPSCs did not give rise to any significant variation in PPF (data not shown). It was therefore reminiscent of transient potentiations observed in older animals after blockade of endocannabinoid dependent- and endocannabinoid-independent components of DHPG-induced suppression of PF-EPSCs. Accordingly, these potentiating effects are likely to be induced at a postsynaptic level in immature and in nearly mature PCs (see DISCUSSION).

In 10- to 12-day-old mice and as in experiments by Maejima et al. (2001), DHPG-induced suppression of PF-EPSCs was nearly totally abolished by 30-min bath application of the CB1 receptor antagonist AM-251 ($n = 9$; Fig. 1B) applied at a saturating concentration of 2 µM (Galley et al. 1996). In 10- to 12-day-old rats, DHPG-induced suppression of PF-EPSCs was similarly abolished ($n = 6$; Fig. 1C) in the presence of the CB1 receptor antagonist SR141716-A (Rinaldi-Carmona et al. 1994) at a saturating concentration of 1 µM (Petitet et al. 1996). In both cases, blockade of agonist-induced suppression of PF-EPSCs by either AM-251 or SR141716-A revealed that the transient potentiation induced by DHPG had an earlier onset than that seen in the absence of CB1 receptor antagonists (Fig. 1, A and B). In 10- to 12-day-old rats, the amplitude and time-course of this potentiation were also slightly, although not significantly, increased in the presence of SR141716-A (Fig. 1C). Altogether, these results confirm that the suppression of PF-EPSCs by DHPG in juvenile rodents depends entirely on retrograde release of endocannabinoids (Maejima et al. 2001).

In nearly mature (18–22 days old) rats ($n = 10$) and for all cells tested, 5-min bath application of 100 µM DHPG induced a transient and significant ($P < 0.001$) decrease in the amplitude of PF-EPSCs that amounted to 43.23 ± 5.08% of control (Fig. 1C). Most importantly, this transient suppression of PF-EPSCs was always accompanied by a significant increase in PPF ($P < 0.001$) from 1.32 ± 0.08 in control conditions to 1.95 ± 0.22 at the peak of the DHPG effect (Fig. 2B). This is in keeping with a presynaptic site of action of DHPG at PF-PC synapses through retrograde signaling in nearly mature rats (Levenes et al. 2001). However, in this earlier study, suppression of PF-EPSCs of similar amplitude as those reported here were obtained with (S)-DHPG concentrations of only 50 µM (compare Fig. 2B1 of this study with Fig. 2B in Levenes et al. 2001). In pilot experiments, suppression of PF-EPSCs induced by 50 µM (S)-DHPG applications was on average 1.5 times smaller than that achieved with the same concentration in this earlier study ($n = 5$; Supplementary Fig. S1). With 25 µM DHPG, the late (endocannabinoid-independent) component of suppression of PF-EPSCs was nearly totally absent and the initial component was still further reduced in amplitude ($n = 5$; Supplementary Fig. S1). This suggests that, taking into account the present recording chamber’s exchange time, this nominal concentration was too low to fully saturate mGlur1 receptors during 5-min bath applications. Therefore concentrations of 100 µM DHPG that gave rise to more robust suppressions of PF-EPSCs were used throughout this study in nearly mature animals. This was also the case in experiments on juvenile rats and mice to compare results with mature ones in the same experimental conditions.

**FIG. 1.** Effects of (S)-3,5-dihydroxyphenylglycine (DHPG) on parallel fiber (PF)-excitatory postsynaptic currents (EPSCs) in control medium and in the presence of CB1 receptor antagonists in juvenile mice (A and B) and rats (C). A: superimposed sweeps of PF-EPSCs elicited in 1 Purkinje cell (PC) by 2 successive PF stimulations (interstimulus interval = 50 ms) in control solution (1), in the presence of 100 µM DHPG (2), and after washout (3) in an 11-day-old mouse. B: plot of mean (±SE; same in all figures except otherwise specified) normalized amplitudes of PF-EPSCs recorded from PCs over time in 10- to 12-day-old mice in control medium (black squares) and in the presence of 2 µM AM-251 (white squares). DHPG (100 µM) was added to the bath for 5 min, as indicated by corresponding horizontal bar. Note that 1, 2, and 3 correspond to numbers indicated in A. C: same plots as in B in 10- to 12-day-old rats in control solution (black squares) and in the presence of 1 µM SR141716-A (white squares).

1 The online version of this article contains supplemental data.
In marked contrast with results obtained in juvenile animals, perfusing nearly mature slices for \( \geq 30 \) min with \( 1 \) \( \mu \)M SR141716-A only partly inhibited the DHPG-induced suppression of PF-EPSCs. Indeed, mean amplitude decrease was only 22.11 \( \pm \) 4.30\% \((n = 14)\), a value significantly smaller \((P < 0.01)\) than that observed in control conditions (Fig. 2B1). The partial inhibitory effect of \( 1 \) \( \mu \)M SR141716-A on the DHPG-induced suppression of PF-EPSCs was unlikely to result from an incomplete blockade of presynaptic CB1 receptors by SR141716-A because bath application of AM-251 at a saturating concentration of \( 2 \) \( \mu \)M did not further antagonize this inhibition of PF-EPSCs. Indeed, in the presence of \( 2 \) \( \mu \)M AM-251, the mean decrease in PF-EPSC amplitude in the presence of \( 100 \) \( \mu \)M DHPG was \( 26 \pm 3.75\% \((n = 6; \text{data not shown})\), a value very similar to that obtained in the presence of \( 1 \) \( \mu \)M SR141716-A. Moreover and in agreement with our previous study (Levenes et al. 1998), these concentrations of SR141716-A and of AM-251 fully antagonized the depressant effect of \( 1 \) \( \mu \)M bath application of the selective CB1 receptor agonist WIN55,212\(\text{--}2\) (Devane et al. 1988) on PF-EPSCs \((n = 3 \text{ in each case; data not shown})\).

Finally, and most importantly, in the presence of CB1 receptor antagonists, the remaining suppression of PF-EPSCs was still accompanied by a significant \((P < 0.01)\) increase in PPF that amounted to 17\%, \(i.e.,\) from 1.29 \( \pm \) 0.05 in control conditions to 1.51 \( \pm \) 0.08 at the peak of the DHPG effect (Fig. 2B2). These latter results suggest that suppression of PF-EPSCs by DHPG in nearly mature rats not only involves activation of presynaptic CB1 receptors like in juvenile animals (Maejima et al. 2001), but also involves at least one other presynaptic mechanism. In contrast, kinetics of PF-EPSCs were not significantly affected. Thus mean values of the 10\(–90\%\) rise time were \(2.06 \pm 0.21\) and \(1.90 \pm 0.20\) ms in control conditions and at the peak of DHPG-induced suppression of PF-EPSCs, respectively, and mean time constants of decay had values of 11.39 \( \pm \) 0.75 and 10.90 \( \pm \) 0.79 ms in the same conditions. Although kinetics are certainly severely biased in nearly mature PCs by dendritic filtering of synaptic currents, these data do not suggest that postsynaptic AMPA receptors at PF-PC synapses are sizably affected during the endocannabinoid-independent component of DHPG-induced suppression of PF-EPSCs (see DISCUSSION).

**Sensitivity of the CB1 receptor--independent component of agonist-induced suppression of PF-EPSCs to a Glu uptake blocker and to a desensitizing KA receptor agonist in nearly mature rats**

In nearly mature rats, retrograde release of Glu by PCs participates in DSE at PF-PC synapses through activation of presynaptic KA receptors that include GluR6 receptor subunits (Crepel 2007). In keeping with this recent finding, the previous study by Levenes et al. (2001) already suggested that activation of postsynaptic mGluR1 decreases PF-EPSCs through retrograde release of Glu and activation of presynaptic ionotropic Glu receptors borne by PFs. Accordingly, in 18- to 22-day-old rats, the endocannabinoid-independent component of agonist-induced suppression of PF-EPSCs should be enhanced in the presence of the Glu uptake inhibitor D-TBOA and suppressed by pharmacological blockade of presynaptic KA receptors.

In the presence of \( 1 \) \( \mu \)M SR141716-A and as expected for a Glu uptake inhibitor, application of \( 100 \) \( \mu \)M D-TBOA increased the amplitude of PF-EPSCs (Fig. 3A1). This increase ranged between 8 and 76\% depending on cells, with a significant mean increase of 34.90 \( \pm \) 9.79\% \((n = 9; P < 0.01)\). As seen in a previous study (Crepel 2007), the rather large variability of potentiating effects of D-TBOA on PF-EPSCs might result from the patterned expression of PC Glu transporters EAAT4 in rat cerebellar cortex (Wadiche and Jahr 2005). In all cells, this effect was accompanied by marked changes in EPSC kinetics (Fig. 3A1), probably because of slower clearance of Glu from synaptic cleft. At the plateau of the D-TBOA effect, application of \( 100 \) \( \mu \)M DHPG decreased the mean amplitude of PF-EPSCs that amounted to 39.78 \( \pm \) 4.63\% of control amplitude before DHPG application (Fig. 3, A2 and B1). This decrease in amplitude was significantly larger \((P < 0.01)\) than that observed in the presence of SR141716-A alone (Fig. 3B1) and was accompanied by a significantly larger increase in PPF \((P < 0.02)\) because PPF increased from 1.24 \( \pm \) 0.17 in SR141716-A + D-TBOA containing medium to 1.77 \( \pm \) 0.15 at the peak of the DHPG effect (Fig. 3B2). Although slower Glu clearance in the presence of D-TBOA may complicate the interpretation of this difference, the fact that D-TBOA alone did not induce any significant change in PPF (Crepel 2007) suggests that D-TBOA did not affect the presynaptic machinery to such an extent as to sizably affect the level of suppression of PF-EPSCs by DHPG. Therefore these results are consistent...
independentsuppression of PF-EPSCs induced by bath application of 100 μM DHPG totally inhibited the late phase of the endocannabinoid-independentsuppression of PF-EPSCs. However, this early SYM 2081-insensitive component and associated PPF increase were partly inhibited by bath application of 300 nM of the GABA_B receptor antagonist CGP55845-A (n = 5; data not shown) and were almost totally blocked when 200 μM of the NO-synthase inhibitor L-NAME (Knowles et al. 1989) was further added to the bath (n = 8; Fig. 4, A and B). This suggests that the early component of the endocannabinoid-independent suppression with the assumption that retrograde release of Glu participates, together with retrograde release of endocannabinoids, to the depressant effect of DHPG on PF-EPSCs in nearly mature rats. However, it should be noted that group 1 mGluRs are not restricted to PCs but are also found, for instance, on glial cells (Angulo et al. 2004; Karakossian and Otis 2004; Parpura et al. 1994).

In keeping with the previous study by Crepel (2007), involvement of presynaptic KA receptors in agonist-induced suppression of PF-EPSCs in 18- to 22-day-old rats was tested by using SYM 2081, a potent ligand that, at micromolar concentrations, selectively blocks KA-induced currents through a process of agonist-induced desensitization (Cho et al. 2003; Cossart et al. 2002; DeVries 2000; Epsztein et al. 2005; Li et al. 1999; Zhou et al. 1997). In the presence of 1 μM SR141716-A, superfusing the slices with 10 μM SYM 2081 did not lead to large changes in PF to PC synaptic transmission, except for a significant (P < 0.001) increase in basal PPF, which amounted to 1.53 ± 0.07% (n = 11) compared with 1.29 ± 0.05% in control conditions. In marked contrast, SYM 2081 totally inhibited the late phase of the endocannabinoid-independent suppression of PF-EPSCs induced by bath application of 100 μM DHPG, and the same was true for the associated increase in mean normalized PPF (n = 11; Fig. 4, A and B). Nearly identical results were obtained in six other cells with 10 μM SYM 2081 + 100 μM d-APV (data not shown). These results strongly suggest that, like for DSE in nearly mature rats, a late phase of the endocannabinoid-independent suppression of PF-EPSCs induced by bath application of 100 μM DHPG involves activation of presynaptic KA receptors.

However, an early component of the DHPG-induced suppression of PF-EPSCs remained unaffected by SYM 2081 and was still accompanied by a significant (P < 0.05) increase in PPF (Fig. 4, A and B). This suggests that one or several other presynaptic components underlie the depressant effect of DHPG on PF-EPSCs. Accordingly, this early SYM 2081-insensitive component and associated PPF increase were partly inhibited by bath application of 300 nM of the GABA_B receptor antagonist CGP55845-A (n = 5; data not shown) and were almost totally blocked when 200 μM of the NO-synthase inhibitor L-NAME (Knowles et al. 1989) was further added to the bath (n = 8; Fig. 4, A and B). This suggests that the early component of the endocannabinoid-independent suppression

FIG. 3. Effect of d-threo-β-benzoyloxyaspartate (d-TBOA) on the CB1 receptor–independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats. A1: superimposed sweeps of PF-EPSCs elicited by 2 successive PF stimulations in the presence of 1 μM SR141716-A and in the presence of 1 μM SR141716-A + 100 μM d-TBOA. A2: as in A1 when 100 μM DHPG was added to the bath and after washout of DHPG. Note the marked effect of d-TBOA on EPSC kinetics. B1: plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares) or of 1 μM SR141716-A + 100 μM d-TBOA (gray squares). Horizontal bar: duration of DHPG application as in Fig. 1. PF-EPSC amplitudes in the presence of 100 μM DHPG, and the same was true for the associated PPF increase. B2: plots of mean PF PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in B1. White lozenges: 1 μM SR141716-A alone; gray lozenges: 1 μM SR141716-A + 100 μM d-TBOA.

FIG. 4. Sensitivity to (2S,AR)-4-methylglutamic acid (SYM 2081) of the CB1 receptor–independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats. A: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares), when 10 μM SYM 2081 was also present in the bath (gray squares), and when 300 nM CGP55845-A + 200 μM L-NAME were further added to the bath (black squares). Horizontal bar: duration of DHPG application as in Fig. 1. B: superimposed plots of mean normalized PF PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in A. White lozenges: 1 μM SR141716-A alone; gray lozenges: 1 μM SR141716-A + 10 μM SYM 2081; black lozenges: 1 μM SR141716-A + 10 μM SYM 2081 + 300 nM CGP55845-A + 200 μM L-NAME.
of PF-EPSCs is caused by activation of presynaptic GABA<sub>B</sub> receptors (Dittman and Regehr 1997) by GABA released by molecular layer inhibitory interneurons, as well as to release of NO from these same cells (Bredt et al. 1990; Shin and Linden 2005; Vincent and Kimura 1992). Indeed, one knows that molecular layer inhibitory interneurons bear group I mGluRs (Baud et al. 1993; Karakossian and Otis 2004) and are therefore likely to be stimulated by DHPG, thus leading to release of GABA and of NO, which in turn transiently depress PF-EPSCs by presynaptic mechanisms (Blond et al. 1997). This presynaptic mGluR1 receptor/NO cascade is reminiscent of the presynaptic N-methyl-D-aspartate (NMDA) receptor/NO cascade found in molecular layer inhibitory interneurons and involved in the induction of cerebellar long-term depression (LTD) through cGMP-dependent inhibition of postsynaptic protein phosphatases (Shin and Linden 2005). Therefore NO released by molecular layer inhibitory interneurons is likely to be involved in both short-term presynaptic and long-term postsynaptic modulation of PF-PC synaptic transmission, depending on additional mechanisms involved, such as activation of postsynaptic mGluR1, protein kinase Cα, and phosphorylation of ser-880 on the AMPA receptor subunit GluR2 for cerebellar LTD (references in Shin and Linden 2005). Finally, blockade of the early component of the endocannabinoid-independent suppression of PF-EPSCs in the eight PCs mentioned above unmasked a short-term potentiation of PF-EPSCs in five of them, whereas no such potentiation was seen in the other three. On average, this potentiation amounted to 130.13 ± 10.11% of control and was accompanied by a slight, although nonsignificant, mean normalized PPF decrease (Fig. 4, A and B). Altogether, these results suggest that, like for DSE (Crepel 2007), a late phase of agonist-dependent suppression of PF-EPSCs in nearly mature rats depends on retrograde release of Glu by PCs and activation of presynaptic KA receptors located on PFs.

**Sensitivity of the CB1 receptor–independent component of suppression of PF-EPSCs by DHPG to nonsaturating concentrations of CNQX and GYKI in nearly mature rats**

Because SYM 2081 is a desensitizing KA receptor agonist rather than a genuine KA receptor antagonist, it was important to confirm the involvement of presynaptic KA receptors in the late phase of agonist-induced suppression of PF-EPSCs. We reasoned that the concentration of Glu achieved at the level of presynaptic KA receptors involved in the CB1 receptor–independent component of agonist-induced suppression of PF-EPSCs is likely to be much lower than that seen by postsynaptic AMPA receptors during PF-PC EPSCs. If so, it is possible that a nonsaturating concentration of the competitive AMPA/KA antagonist CNQX (Honoré et al. 1988) that only partly block PF-EPSCs is sufficient to fully antagonize presynaptic KA receptors and thus inhibit agonist-induced suppression of PF-EPSCs.

Indeed, bath application of 1 μM CNQX in the presence 1 μM SR141716-A reduced the amplitude of PF-EPSCs to 36.33 ± 3.76% of the control value (n = 11). Unexpectedly, this effect was accompanied by a large and highly significant (P < 0.001) PPF increase, from 1.30 ± 0.08 to 1.75 ± 0.14 (Fig. 5 A1) that was reminiscent of that observed for basal PPF in the presence of SYM 2081 in nearly mature rats and in GluR<sub>6</sub>−/− mice (Crepel 2007). In keeping with this latter result that suggests a presynaptic site of action of CNQX in addition to its well-established postsynaptic effect, mean CV of PF-EPSCs also significantly (P < 0.001) increased from to 0.045 ± 0.006 in control to 0.081 ± 0.012 at the steady state of the depressant effect of CNQX (n = 9; Fig. 5B).

Like for SYM 2081, CNQX also markedly inhibited the late phase of the CB1 receptor–independent suppression of PF-EPSCs induced by bath application of 100 μM DHPG (Fig. 5A2). Moreover, its initial phase was also significantly (P < 0.01) inhibited because the mean decrease in peak amplitude of
PF-EPSCs was only 11.09 ± 2.25% (n = 11) compared with 22.11 ± 4.30% (n = 14) in the presence of SR141716-A alone (Fig. 5A2). This near 50% inhibition of the initial phase of the agonist-induced suppression of PF-EPSCs by CNQX was accompanied by a similar near 50% inhibition of associated PPF increase. As such, the remaining 9% PPF increase, from 1.75 ± 0.14 in the presence of CNQX alone to 1.91 ± 0.15 at the peak of the residual depressant effect of DHPG, was no longer significant (Fig. 5A1).

To preclude that the strong reduction in PF-EPSC amplitude by CNQX was not solely responsible for the lack of suppression of PF-EPSCs by DHPG in the experiments reported above, we also studied the effect of nonsaturating concentrations of AMPA receptor antagonist (Bureau et al. 1999; Renard et al. 1995; Wilding and Huettner 1995). On average, bath application of 20 μM GYKI reduced the amplitude of PF-EPSCs to 42.63 ± 3.92% of control values (n = 11), a decrease nonsignificantly different from that obtained with 1 μM CNQX. Like for experiments with CNQX, this decrease in EPSC amplitude was also accompanied by a significant (P < 0.01) PPF increase, although about only one half of that obtained in the presence of CNQX (Fig. 5A1). However, and in marked contrast with results obtained with CNQX, mean CV of PF-EPSCs did not significantly increase during the depressant effect of GYKI on PF-EPSCs, because mean CV values during the control period and at the steady state of the effect of GYKI were 0.046 ± 0.005 and 0.051 ± 0.006, respectively (Fig. 5B).

In 8 of the 11 tested cells, GYKI did not significantly inhibit the CB1 receptor–independent suppression of PF-EPSCs induced by bath application of 100 μM DHPG, because the mean decrease in peak amplitude of PF-EPSCs was 28.48 ± 7.07% compared with 22.11 ± 4.30% (n = 14) in the presence of SR141716-A alone (Fig. 5A2). Moreover, the time-course of PF-EPSC suppression was unchanged (cf. Fig. 2B1 and 5A2). In the remaining three cells, GYKI totally abolished the CB1 receptor–independent suppression of PF-EPSCs induced by bath application of 100 μM DHPG, which unmasked a short-term potentiation of PF-EPSCs (Fig. 5A2). Because there was no apparent difference between these two groups of cells with respect to the effect of GYKI on PF-EPSC amplitude and on basal PPF, DHPG results were pooled, leading to a mean suppressing effect of this compound on peak amplitude of PF-EPSCs that amounted to 20.83 ± 5.76%. This value was very close to that obtained in the presence of SR141716-A alone. Accordingly, DHPG suppression of PF-EPSCs was still accompanied by a significant (P < 0.05) and near 20% increase in mean PPF, from 1.54 ± 0.08 in the presence of SR141716-A + GYKI to 1.82 ± 0.18 at the peak of DHPG effect (Fig. 5A1).

Together, CNQX and GYKI results fully confirm that presynaptic KA receptors are likely to be involved in CB1 receptor–independent suppression of PF-EPSCs by mGluR1 agonists (see DISCUSSION).

Finally, because PF-EPSC amplitude did not reach a steady state during DHPG application (Figs. 2B1 and 5A2), no attempt was made to apply the CV method here. In contrast, such a near steady state was achieved during the endocannabinoid-independent component of DSE (Crepel 2007) and was accompanied by a significant increase in CV (see Supplementary Results and Supplementary Fig. S2), thus confirming involvement of presynaptic mechanisms in the CB1 receptor–independent component of DSE (Crepel 2007).

DHPG sensitivity of PF-EPSCs in wild-type and GluR6−/− mice

Presynaptic KA receptors located on PFs are likely to be heteromeric constructions that include GluR6 and KA2 receptor subunits (Lerma et al. 2001; Petralia et al. 1994). Their activation up- or down-regulates Glu release depending on agonist concentration (Delaney and Jahrl 2002). Agonist-induced suppression of PF-EPSCs was studied in nearly mature GluR6−/− mice and compared with that of wild-type mice of the same strain (see METHODS), with the assumption that invalidating GluR6 receptor subunits renders presynaptic KA receptors nonfunctional (Ruiz et al. 2005). All experiments in GluR6−/− mice were performed in the presence of 50 μM D-APV to minimize possible developmental compensations by presynaptic NMDA receptors and in the presence of 1 μM SR141716-A to focus on the endocannabinoid-independent component of agonist-induced suppression of PF-EPSCs. For comparison, all experiments in wild-type mice were also performed in the presence of 50 μM D-APV and of 1 μM SR141716-A.

In 22- to 24-day-old wild-type mice, 5-min bath application of 100 μM DHPG induced an endocannabinoid-independent suppression of PF-EPSCs of similar amplitude and duration as in nearly mature rats, with a mean peak amplitude decrease of 17.62 ± 2.76% (n = 6; Fig. 6A). This suppression was accompanied by a significant (P < 0.001) increase in mean normalized PPF, from 100.39 ± 3.66% in control conditions to 116.27 ± 3.24% at the peak of the DHPG effect (Fig. 6B).

In 22- to 24-day-old GluR6−/− mice and in keeping with a previous study (Crepel 2007), no major alteration in PF to PC synaptic transmission was seen, except for basal PPF, which amounted to 1.54 ± 0.01% (n = 6) compared with 1.31 ± 0.05% in control mice, the difference being highly significant (P < 0.001). In contrast, the late phase of the endocannabinoid-independent suppression of PF-EPSCs by DHPG and of its associated increase in PPF was strongly inhibited in all cells, whereas the initial phase remained largely unaffected (Fig. 6, A and B). Therefore these results are very similar to those obtained in experiments performed in the presence of SYM 2081 and of CNQX and fully confirm that the late phase of the endocannabinoid-independent suppression of PF-EPSCs by DHPG depends on retrograde release of Glu. Moreover, they suggest that Glu acts through activation of KA receptors that include GluR6 subunits.

**GDP-βS sensitivity of the CB1 receptor–independent component of agonist-induced suppression of PF-EPSCs in nearly mature rats**

As mentioned before, group 1 mGluRs are not restricted to PCs but are also found on various cell types including granule cells, molecular layer interneurons, and glial cells (Angulo et al. 2004; Baude et al. 1993; Parpura et al. 1994). As such, glutamate release from these cells might participate in DHPG-induced suppression of PF-EPSCs. In an attempt to exclude this possibility, we selectively blocked G protein activity in the postsynaptic compartment by dialyzing PCs through the patch clamp and applying GDP-βS to the medium. In a preliminary study, an increase in PPF was observed in both wild-type and GluR6−/− mice (Fig. 7B).

**Conclusion**

The present study demonstrates that CB1 receptor–independent suppression of PF-EPSCs in near maturity is mediated by endogenous cannabinoids acting via presynaptic CB1 receptors and not through any other cannabinoid receptor. Such suppression is highly specific and unique to the endocannabinoid-sensitive component of DSE observed in wild-type mice but not in the GluR6−/− mice. The suppression is of similar amplitude and duration as that observed in the presence of SR141716-A, a selective CB1 receptor antagonist. This effect is mediated by both mGluR1 and mGluR5, and is strongly inhibited by the mGluR antagonist (S) 8-CPT-cGMP and also by R(+)-iCys, a selective mGluR5 agonist. In contrast, the late phase of the endocannabinoid-independent suppression of PF-EPSCs by DHPG depends on retrograde release of Glu. Moreover, they suggest that Glu acts through activation of KA receptors that include GluR6 subunits.
pipette for ≥30 min after break-in with a conventional K-gluconate internal solution with 4 mM GDP-S added (Galante and Diana 2004); GDP-S is a nonhydrolyzable GTP analog that inhibits G protein activity. Experiments were performed in the presence of 1 µM SR141716-A to focus on the endocannabinoid-independent suppression of PF-EPSCs. In five of the six cells tested, the late phase of the endocannabinoid-independent suppression of PF-EPSCs induced by bath application of 100 µM DHPG was abolished and replaced by a short-term potentiation of PF-EPSCs, whereas its initial phase was much less affected (Fig. 7A). In these five cells, the late phase of mean normalized PPF increase associated with the endocannabinoid-independent suppression of PF-EPSCs was also inhibited and replaced by a nonsignificant 7.72 ± 2.83% decrease of the mean normalized PPF (Fig. 7B). In the remaining PC, the late phase of the endocannabinoid-independent suppression of PF-EPSCs was only partly abolished (data not shown). In contrast, in five other PCs dialyzed during the same period of time after break-in with a conventional K-gluconate internal solution without GDP-BS, a clear-cut endocannabinoid-independent suppression of PF-EPSCs and associated

These results strongly suggest that the Glu release responsible for the late phase of mGluR1-induced suppression PF-EPSCs originates mainly from the recorded PC themselves, with only minor (if any) contribution of spillover arising from neighboring cells.

**Ryanodine sensitivity of the CB1 receptor–independent component of agonist-induced suppression of PF-EPSCs and of DSE in nearly mature rats**

Depolarization-induced potentiation of inhibition (DPI) also operates through Glu release from depolarized PCs. In this case, Glu activates presynaptic NMDA receptors, resulting in a slow build-up and decay (over several minutes) of calcium release from presynaptic ryanodine-sensitive calcium stores (Duguid and Smart 2004). As for DPI, the KA-dependent components of agonist-induced suppression of PF-EPSCs and
of DSE at PF-PC synapses might therefore involve such a mechanism in nearly mature rats, as suggested previously (Crepel 2007; but see also Carter et al. 2002). This hypothesis was tested by the following experiments.

In the presence of 1 μM SR141716-A and of 100 μM ryanodine, the CB1 receptor–independent suppression of PF-EPSCs by bath application of 100 μM DHPG was strongly inhibited and replaced by a transient potentiation of PF-EPSCs, 112.84 ± 9.43% on average (n = 6; Fig. 8A1). In these cells, PPF increase associated with this CB1 receptor–independent suppression of PF-EPSCs was also strongly inhibited (Fig. 8A2). Interestingly, ryanodine also markedly reduced basal PPF because its mean value was only 1.09 ± 0.06 (n = 6; Fig. 8A2), thus suggesting that presynaptic ryanodine-sensitive calcium stores also contribute to basal PPF at PF-PC synapses in nearly mature rats.

In the same bathing medium, the late phase of the CB1 receptor–independent suppression of PF-EPSCs induced by PC depolarization (from −70 to 0 mV for 1 s) was also strongly inhibited, as well as the associated mean normalized PPF increase (n = 6; data in the presence of SR141716-A alone taken from Crepel 2007; Fig. 8, B1 and B2). Here again, bath application of 100 μM ryanodine also markedly reduced basal PPF because the mean paired-pulse ratio was only 0.90 ± 0.04 in these experiments (n = 6). The fact that the early component of DSE that was partly resistant to bath application of ryanodine was not accompanied by significant changes in paired-pulse ratio (Fig. 8, B1 and B2) suggests in turn that it might be partly postsynaptic in origin, i.e., caused by a transient ionic depolarization (from receptor–independent suppression of PF-EPSCs induced by PC depolarizing pulses from −70 to 0 mV in another series of DSE experiments in the presence of ryanodine. Indeed, this duration was likely to give rise to postsynaptic calcium transients of similar amplitude to those observed with 1-s depolarizing pulses in the absence of ryanodine (Fig. 9B1). As shown in Fig. 8B1, the Glu-dependent component of DSE (Crepel 2007) was still significantly inhibited (n = 9; P < 0.01), whereas the early residual DSE was not significantly different from that induced in the same conditions with 1-s depolarizing pulses (Fig. 8B1).

Fluorometric experiments were also performed to distinguish between pre- and postsynaptic ryanodine effects on DSE to avoid as much as possible slow time resolution inherent to conventional bath application of mGluR1 agonists. More specifically, we tested the effects of 100 μM bath applied ryanodine on the amplitude and time-course of calcium transients induced in PCs by a depolarizing voltage step from −70 to 0 mV for 1 s on the one hand and induced

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**FIG. 8.** Sensitivity to ryanodine of agonist-induced suppression of PF-EPSCs and of depolarization-induced suppression of excitation (DSE) in nearly mature rats. A1: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares) or of 1 μM SR141716-A + 100 μM ryanodine (black squares) before, during, and after 100 μM DHPG was further added to the bath, as indicated by the corresponding horizontal bar. A2: superimposed plots of mean PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in A1. White lozenges: 1 μM SR141716-A alone; black lozenges: 1 μM SR141716-A + 100 μM ryanodine. B1: superimposed plots of mean normalized amplitudes of PF-EPSCs recorded from PCs over time in the presence of 1 μM SR141716-A alone (white squares; data taken from Crepel 2007) or of 1 μM SR141716-A + 100 μM ryanodine before and after a depolarizing pulse from −70 to 0 mV for 1 s (black squares), applied at time 0 (arrow). Plot with gray squares: same as plot with black squares, except that depolarizing pulse duration was 2 s. B2: superimposed plots of mean normalized PPF of PF-EPSCs recorded in the same conditions and from the same PCs as in B1. White lozenges: 1 μM SR141716-A alone; black lozenges: 1 μM SR141716-A +100 μM ryanodine.

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Strongly suggest that prolonged calcium release from presynaptic ryanodine-sensitive stores occurs in PFs after brief activation of presynaptic KA receptors.

In control bathing medium, calcium signals induced in PC dendrites by the above described DSE protocol (depolarizing voltage steps from −70 to 0 mV for 1 s) peaked to ∆F/F = 14.10 ± 2.85% at the end of the depolarizing steps (Fig. 9B1) and rapidly decayed over the course of 10–15 s (n = 6; Fig. 9B2). Superfusion of the slices for 30 min with 100 μM ryanodine significantly (P < 0.05) inhibited these depolarization-induced calcium transients because mean ∆F/F was now only 7.77 ± 1.63% (n = 6; Fig. 9B1 and B2). Differences between control and ryanodine results were further quantified by determining again, for each cell, mean ∆ values calculated from the start of the depolarizing step until 4 s after, i.e., the period of time during which ∆F/F plots in control conditions and in the presence of ryanodine appeared clearly different (Fig. 9B2). The mean ∆ value was significantly lower (P < 0.05) than ryanodine (4.56 ± 1.17) than in its absence (8.52 ± 1.27). Here again and in keeping with previous results by Carter et al. (2002), this suggests that calcium release from intracellular ryanodine-sensitive stores participates in calcium transients induced in PC dendrites by short depolarizing voltage steps identical as those used to induce DSE (Crepel 2007). However, these calcium transients were much shorter than those induced in PFs by domoate application, and moreover, they were also much shorter than the duration of the KA-sensitive component of DSE in nearly mature rats (Crepel 2007). Therefore electrophysiological and fluorometric experiments with ryanodine clearly suggest that prolonged calcium release from presynaptic ryanodine-sensitive stores is responsible for the prolonged duration of DSE and of agonist-induced suppression of PF-EPSCs in nearly mature rats.

**Origin of the lack of the Glu-dependent component of agonist-suppressed depression of PF-EPSCs in immature rats**

Depolarization-induced potentiation of inhibition that operates through Glu release from depolarized PCs is strictly dependent on the activity of surrounding Glu transporters (Duguid and Smart 2004). Therefore the lack of a Glu-dependent component of agonist-suppressed suppression of PF-EPSCs in juvenile animals might simply result from an unbalance between Glu release and Glu uptake in favor of the latter at early developmental stages. In 10- to 12-day-old rats and in the presence of 1 μM SR141716-A, bath application of the Glu uptake inhibitor d-TBOA (100 μM) did not unmask any sizeable Glu-dependent component of agonist-suppressed depression of PF-EPSCs (n = 5; Fig. 10A). However, the transient potentiation elicited by DHPG was significantly (P < 0.001) inhibited compared with that observed in the presence of SR141716-A alone (Fig. 10A), suggesting that it was counter-balanced by a DHPG-induced suppression of PF-EPSCs of similar amplitude and time-course. Thus and as shown for DSE (Crepel 2007), the absence of a Glu-dependent component of agonist-induced depression of PF-EPSCs in juvenile rats may be partly explained by the activity of surrounding Glu transporters. However, this absence might also be caused by other factors such as incomplete maturation of presynaptic KA receptors or of presynaptic calcium-induced calcium release, because d-TBOA failed to reveal any fully developed KA-
dependent component of agonist-induced suppression of PF-EPSCs in these animals. Therefore fluorometric experiments were also performed to test these hypotheses.

In 12-day-old rats and like in experiments in nearly mature rats, PFs loaded with Fluo-4FF-AM were subjected to focal superfusion of 20 μM domoate for 1 min. Here again, this elicited a transient increase in cytosolic calcium signals in these fibers, which peaked shortly after the end of domoate application (n = 5; Fig. 10B1). However, these calcium transients were significantly (P < 0.001) smaller than in nearly mature rats in the same conditions, because the mean peak \( \Delta F/F \) was only 1.82 ± 0.21%. Moreover, calcium transients relaxed within <2 min instead of within 30 min in nearly mature rats (cf. Figs. 9A2 and 10B1). Therefore these results are in agreement with the lack of a Glu-dependent component of agonist-induced suppression of PF-EPSCs in immature rats and suggest that presynaptic KA receptors and/or calcium release from internal stores are not yet fully developed at this early developmental stage.

In contrast, when domoate application was performed in the presence of 100 μM D-TBOA, cytosolic calcium signals were significantly (P < 0.001) larger (\( \Delta F/F = 4.74 \pm 0.53\% \)) and now decayed over 15 min (n = 5; Fig. 10B1). Moreover, this decay was markedly shortened when 100 μM ryanodine was also present in the bath (n = 5; Fig. 10B1), so that the mean \( \delta \) value was significantly lower (P < 0.05) in the presence of ryanodine (0.96 ± 0.30) than in its absence (2.65 ± 0.47). These results therefore suggest that at least some calcium-induced calcium release may be triggered in immature PFs.

However, the prominent effect of D-TBOA on cytosolic calcium signals was puzzling. Indeed, and to the best of our knowledge, significant uptake of this agonist is unlikely. However, domoate is known to induce release of excitatory amino acids from cultured cerebellar granule cells through reversal of the Glu transporter (Berman and Murray 1997). Therefore the possibility exists that, in these experiments, domoate not only acts through activation of presynaptic KA receptors, but also activates other presynaptic Glu receptors after release of Glu from the superfused molecular layer. We therefore tested this possibility by studying the effect of bath-applied D-APV (50 μM) on calcium transients elicited in PF by focal superfusion of the slices for 1 min with 20 μM domoate in the presence of D-TBOA. We performed these experiments because NMDA receptors are characterized by their high affinity for Glu and are probably present and functional on PFs (Casado et al. 2000). Furthermore, these results also strongly suggest that activation of NMDA receptors is necessary to establish the aforementioned hypothesis, and also supports the previous finding that NMDA receptors are present on PFs (Casado et al. 2000). However, this conclusion has been challenged recently (Shin and Linden 2005) and, to date, it still has not been established by electron microscopic analysis that NMDA receptors exist on PFs. As shown in Fig. 10B2, D-APV shortened calcium transients induced by domoate in the presence of D-TBOA (n = 6) to nearly the same extent as that observed with ryanodine because mean \( \delta \) values were not significantly different, i.e., 0.96 ± 0.18 and 0.96 ± 0.30, respectively. This latter result indicates that activation of presynaptic NMDA receptors probably contributes to calcium transients induced by domoate in the presence of Glu uptake blockers, thus corroborating the aforementioned hypothesis, and also supports the previous finding that NMDA receptors are present on PFs (Casado et al. 2000). Furthermore, these results also strongly suggest that activation of NMDA receptors is necessary to trigger calcium-induced calcium release in immature PFs. In contrast, D-APV did not significantly affect calcium transients induced by domoate in control bathing medium in 20- to 22-day-old rats (n = 4; data not shown), suggesting that presynaptic NMDA receptors are not recruited in this case.
In 12-day-old rats, the mean \( \bar{d} \) values of calcium transients elicited in the presence of \( \beta \)-TBOA and \( \beta \)-APV or in the presence of \( \beta \)-TBOA and ryanodine were still significantly \( (P < 0.05) \) higher \( (0.96 \pm 0.18 \) and \( 0.96 \pm 0.30 \), respectively) than in control medium \( (0.10 \pm 0.02; \text{Fig. 10B2}) \). It is therefore likely that an additional mechanism participates to calcium transients induced by domoate in the presence of \( \beta \)-TBOA. One hypothesis is that Glu uptake blockers lead to a progressive accumulation of Glu within slices that tonically activates granule cells. Such activity might therefore increase nal calcium concentration within PFs and facilitate induction of calcium-induced calcium release on activation of presynaptic KA receptors. If this were so, prolonged calcium transients induced by domoate in the presence of \( \beta \)-TBOA and \( \beta \)-APV should be no longer observed in the presence of TTX. However, in six experiments performed in the presence of \( 1 \mu M \) TTX, calcium transients induced by focal application of \( 20 \mu M \) domoate in the presence of both \( 100 \mu M \beta \)-TBOA and \( 50 \mu M \beta \)-APV was not significantly altered compared with that induced when TTX was omitted (Fig. 10B3). At the moment, we have no other plausible explanation to explain that calcium transients elicited in the presence of \( \beta \)-TBOA and \( \beta \)-APV or in the presence of \( \beta \)-TBOA and ryanodine were still significantly larger and of longer duration than in control medium.

**Discussion**

In keeping with the study by Levenes et al. (2001) and Crepel (2007), these data strongly suggest that, in nearly mature rats, retrograde release of Glu is involved in the suppression of synaptic transmission at PF-PC synapses after activation of postsynaptic mGluR1. Moreover, results with GDP-\( \beta \)S are consistent with the view that Glu mainly originates from the recorded PC themselves, with only minor (if any) contribution of spillover arising from neighboring cells. As for the KA-dependent component of DSE at PF-PC synapses in nearly mature rats (Crepel 2007), Glu is likely to operate through activation of presynaptic KA receptors located on PFs and prolonged calcium release from presynaptic ryanodine-sensitive calcium stores. Finally, results in nearly mature wild-type and GluR6\(^{-/-}\) mice further suggest that, as for DSE (Crepel 2007), presynaptic KA receptors involved in agonist-induced suppression of PF-EPSCs include GluR6 receptor subunits. These results also show that retrograde release of endocannabinoids is another major component in agonist-induced suppression of excitation at these nearly mature PF-PC synapses. In contrast, in juvenile rats and mice, suppression of synaptic transmission at PF-PC synapses by activation of postsynaptic mGluR1 is entirely mediated through retrograde release of endocannabinoids by PCs and activation of presynaptic CB1 receptors. Our results are in agreement with those previously obtained in juvenile animals for agonist-induced suppression of PF-EPSCs (Maejima et al. 2001) and for DSE (Brenowitz and Regehr 2003; Crepel 2007; Kreitzer and Regehr 2001b).

**Suppression of PF-EPSCs by DHPG in juvenile rats and mice depends entirely on retrograde release of endocannabinoids**

In juvenile rats and mice, DHPG-induced suppression of PF-EPSCs exclusively involved retrograde release of endocannabinoids by PCs and activation of presynaptic CB1 receptors. This is in complete agreement with previously published results in juvenile mice by Maejima et al. (2001). The lack of any detectable Glu-, GABAB-, or NO-dependent components of suppression of PF-EPSCs by DHPG in juvenile animals suggests in turn that these pathways are likely to mature later than endocannabinoid signaling. However, the present results (see above) also strongly suggest that the absence of endocannabinoid-independent suppression of PF-EPSCs by DHPG in juvenile rats is partly caused by an unbalance between Glu release and Glu uptake in favor of the latter at early developmental stages, coupled with either immature presynaptic KA receptors and/or not fully developed calcium release from ryanodine-sensitive internal stores.

In this study and in that of Maejima et al. (2001) in juvenile animals, a transient potentiation effect of DHPG on PF-EPSC was observed, in particular in the presence of CB1 receptor antagonists. This was also true in nearly mature synapses when all pathways of the DHPG suppressive effect on PF-EPSCs were blocked (see **Results**). This potentiation might be of postsynaptic origin because it was not accompanied by any significant change in PPF. In keeping with the potentiating effect of mGluR1 agonists on PC responsiveness to ionophoretic application of Glu in their dentritic field (Levenes et al. 2001). Further studies will have to unravel underlying mechanisms of this transient potentiation of PF-EPSCs.

Therefore sequential development of mechanisms underlying short-term plasticity at PF-PC synapses may at least partly explain apparent contradictions between data by Levenes et al. (2001) and by Maejima et al. (2001).

**DHPG suppression of PF-EPSCs in nearly mature rats depends partly on retrograde release of glutamate and activation of presynaptic KA receptors**

In nearly mature rats, suppression of PF-EPSCs by DHPG was only partly blocked by CB1 receptor antagonists. Moreover, the remaining component was potentiated by Glu uptake inhibitors and markedly inhibited by the desensitizing KA receptor agonist SYM 2081 and by nonsaturating concentrations of the competitive AMPA/KA receptor antagonist CNQX (Honoré et al. 1988). However, this inhibitory effect was not reproduced by nonsaturating concentrations of the more selective AMPA receptor antagonist GYKI (Bureau et al. 1999; Renard et al. 1995; Wilding and Huettner 1995). These results strongly suggest that the depressant effect of DHPG on PF-EPSCs not only involves retrograde release of endocannabinoids as in immature rats and mice, but also involves retrograde release of Glu by PCs and subsequent activation of KA receptors. Moreover, and as mentioned above, results in wild-type and GluR6\(^{-/-}\) mice further suggest that these KA receptors include GluR6 subunits. Finally, the nearly complete blockade of endocannabinoid-independent suppression of PF-EPSCs with GDP-\( \beta \)S makes unlikely that Glu responsible for this suppression might be spilling over in significant concentrations from neighboring cellular elements such as glial cells (Angulo et al. 2004; Parpura et al. 1994).

The fact that the endocannabinoid-independent suppression of PF-EPSCs by mGluR1 agonists was still accompanied by an increase in PPF that itself was potentiated by Glu uptake inhibitors and markedly inhibited by SYM 2081 as well as by...
nonsaturating concentrations of CNQX further suggests that, like for DSE in nearly mature rats (Crepel 2007), KA receptors involved in agonist-induced suppression of PF-EPSCs are located on PFs. In contrast, the absence of significant modification of PF-EPSC kinetics during the endocannabinoid-independent suppression of PF-EPSCs (see Results) suggests that released Glu does not reach postsynaptic AMPA receptors in significant concentrations, probably because of their lower sensitivity to glutamate than KA receptors (Lerma et al. 2001) and retarded transmitter diffusion around synaptic spines (Barbour et al. 1994).

Finally, DHPG suppression of PF-EPSCs in nearly mature rats is also likely to involve activation of presynaptic GABA<sub>B</sub> receptors and release of NO from molecular layer inhibitory interneurons (see Results). Such a complexity of effects pertaining to DHPG may well have led to oversight of the CB1 receptor–dependent component of the PF-EPSC suppression induced by mGluR1 agonists in the previous study by Levenes et al. (2001).

**Effect of nonsaturating concentrations of CNQX and of GYKI on PPF and CV**

In a previous study, Delaney and Jahr (2002) showed that weak activation of presynaptic KA receptors borne by PFs up-regulate Glu release by these fibers. This fits well with these results showing that nonsaturating concentrations of CNQX increase basal PPF and CV. Indeed, if one assumes that the basal extracellular Glu concentration is sufficient to induce weak tonic activation of presynaptic KA receptors that in turn increase release probability at PF-PC synapses, reversal of such an effect by CNQX will effectively lead to the observed increases in basal PPF and CV. Similarly, Delaney and Jahr (2002) showed that larger activation of presynaptic KA receptors borne by PFs down-regulates Glu release by these fibers. As such, the larger Glu concentration achieved through retrograde Glu release during DSE or during agonist-induced suppression of PF-EPSCs is now likely to down-regulate Glu release at these synapses, perhaps through depletion of the readily releasable pool of synaptic vesicles (Crepel 2007; Levenes et al. 2001), explaining the associated PPF increase.

In this study, the increase in basal PPF induced by nonsaturating concentrations of GYKI raises a question, in particular because it was not accompanied by a corresponding increase in mean CV, at least when considering the first EPSCs within PF-EPSC pairs in PF experiments. Accordingly, and even though this PPF increase was only one half of that induced by nonsaturating concentrations of CNQX causing similar reduction in PF-EPSC amplitude (see Results), it remains difficult to interpret these results solely on the basis of a partial antagonist effect of GYKI on presynaptic KA receptors that contain GluR6 subunits (Bureau et al. 1999). Indeed, this PPF increase was observed in all tested cells, whereas the strong inhibition by GYKI of the effect of DHPG on PF-EPSCs only concerned less than a third of them (see Results). Among other possible explanations for such a puzzling effect of GYKI, one can point to potassium efflux that occurs through postsynaptic AMPA receptors during PF-EPSCs. In PF experiments, if one assumes that clearance of potassium released during pairs of EPSCs is incomplete when the next pair is elicited (PF stimulations at 0.33 Hz), such a residual increase in extracellular potassium concentration might contribute to steady depolarization of active zones, thereby leading to a decrease of basal PPF. Reversal of this effect by strong blockade of postsynaptic AMPA receptors by GYKI would therefore reduce release probability of Glu during EPSCs and thus explain the observed 16% basal PPF increase in these experiments. In keeping with this hypothesis, we nearly always noticed a progressive increase in PF-EPSC amplitude and a correlative progressive decrease of associated PPF during the first minutes of PF stimulations, before reaching a quasi steady state (see Fig. 5A).

Along the same line, increasing the frequency of PF stimulation from 0.1 to 0.33 Hz decreased within a few minutes basal PPF from 1.94 ± 0.18 to 1.42 ± 0.20 in the four tested cells, and this effect was accompanied by a correlative increase in amplitude of PF-EPSCs. Noteworthy, CV seemed more reliable than PPF in establishing that GYKI suppresses PF-EPSCs by selectively blocking postsynaptic AMPA receptors in these experiments because this depressant effect was not accompanied by any significant correlative increase in CV (see Results). Following this interpretation, only about one half of the basal PPF increase accompanying inhibition of PF-EPSCs by nonsaturating concentrations of CNQX would be genuinely caused by its blocking effect on presynaptic KA receptors, in agreement with the correlative increase in CV.

Finally, it must be emphasized that the ratio $r = \frac{\text{mean basal PPF increase/mean EPSC amplitude decrease during superfusion of the slices with GYKI}}{\text{0.29 compared with 0.97 and 0.76, respectively, for the ratio } R = \frac{\text{mean PPF increase/mean EPSC amplitude decrease during DHPG application in the presence of SR141716-A + GYKI}}{\text{and 0.76, respectively, for the ratio } R = \frac{\text{mean PPF increase/mean EPSC amplitude decrease during superfusion of the slices with GYKI}}{\text{SR141716-A alone (see Results)}}}$.

Therefore the mechanism proposed above to explain the unexpected effects on PPF of blocking postsynaptic AMPA receptors by GYKI can hardly explain the bulk of the PPF increase accompanying suppression of PF-EPSCs by mGluR1 agonists. The latter remains therefore most likely caused by activation of presynaptic KA receptors by Glu released by PCs. Concerning the KA-dependent component of DSE, the ratio $R$ was close to 0.5 on average (Crepel 2007), so that it was no longer possible, by simply comparing $r$ and $R$ values, to exclude that a significant contribution of prolonged clearance of potassium after PF-EPSCs participates in the observed PPF increase. However, the KA-dependent component of DSE was also accompanied by a significant increase in CV and, moreover, its prolonged time-course closely matched that of presynaptic ryanodine-sensitive calcium transients induced by short domoate application to PFs (see Results). Therefore here again, PPF and CV increases accompanying DSE of PF-EPSCs are most likely primarily caused by activation of presynaptic KA receptors by Glu released by PCs. On the whole, these data are also consistent with earlier reports on dendritic release of Glu from neocortical neurons (Ali et al. 2001; Harkany et al. 2004).

**Contribution of pre- and postsynaptic ryanodine-sensitive calcium stores to the KA-dependent components of DSE and to agonist-induced suppression of PF-EPSCs in nearly mature rats**

Results of fluorometric experiments and those showing a nearly complete inhibition of the KA-dependent components of DSE and of agonist-induced suppression of PF-EPSCs by
ryano-dine suggest that their long duration is caused by pro-
longed calcium release from presynaptic ryanodine-sensitive
calcium stores, after initial activation of presynaptic KA re-
ceptors by Glu released by PCs. In particular, no prolonged
ryanodine-sensitive calcium transient could be observed in PC
dendrites after the same depolarizing pulses applied to PC
soma as those used to induced DSE, whereas such prolonged
ryanodine-sensitive calcium transients were readily elicited
within PFs by focal application of low concentrations of the
selective KA agonist domoate (see RESULTS). Because of tech-
nical constraints (see METHODS), we used focal applications
of domoate lasting 60 s instead of only 4 s as in experiments by
Duguid and Smart (2004). This may well explain why the
duration of presynaptic ryanodine-sensitive calcium transients
in these experiments was about twice that of inhibitory synap-
tic noise elicited in PCs by brief activation of presynaptic
NMDA receptors (Duguid and Smart 2004) and also nearly 3
times longer than duration of the KA-dependent component of
DSE (see Fig. 8B1) (Crepel 2007).

In addition, these results also suggest that presynaptic ryan-
odine-sensitive calcium stores participate in the residual cal-
cium increase responsible for basal PPF at PF-PC synapses
because ryanodine also markedly reduced basal PPF values
(see RESULTS). Finally, one cannot totally exclude that calcium
release from postsynaptic ryanodine-sensitive calcium stores
(Carter et al. 2002; Isokawa and Alger 2006) also participates
to retrograde release of Glu from PCs. However, this contri-
bution is unlikely to be important because, in DSE experiments
in the presence of ryanodine, compensation for inhibition of
postsynaptic calcium-induced calcium release by increasing
postsynaptic stimulation failed to restore normal DSE (see
RESULTS).

It must be acknowledged that these results seem to contra-
dict previous observations by Carter et al. (2002), who con-
cluded that presynaptic ryanodine-sensitive calcium stores do
not contribute to synaptic transmission at PF-PC synapses in
10- to 22-day-old rats. However, and interestingly enough,
these authors suggested that internal calcium stores may well
play a role in presynaptic function at later developmental
stages as is the case for granule cells and their associated
parallel fibers in the avian cerebellum where ryanodine recep-
tors are only prominent in mature animals (Ouyang et al.
1997). Therefore the possibility remains that subtle age differ-
ces of animals under study explain the apparent contradic-
tion between the two set of results because, in particular, ages
of rats in these experiments correspond to the upper limit of
those included in that of Carter et al. (2002).

Finally, the mechanisms described here could take place in
physiological conditions and have functional consequences.
Indeed, one knows that postsynaptic mGluR1 are activated by
high-frequency activity of PFs (Batchelor et al. 1994) and that
the prolonged discharge of excitatory quantal events that fol-
low short PF tetanus is sensitive to the mGluR1 antagonist
AIDA and to the KA receptor blocker SYM 2081 (Crepel
2007; Levenes et al. 2001). Thus if one assumes that this
Glu-dependent discharge of excitatory quantal events is suffi-
cient to trigger action potentials along these fibers as previ-
ously suggested (Levenes et al. 2001), this would introduce a
form of local communication among PCs sharing the same PF
input. However, in these experiments, this enhanced discharge
was terminated a few seconds after PF tetanus, whereas in this
study, the duration of the KA-sensitive component of suppres-
sion of PF-EPSCs by DHPG outlasted its washout by several
minutes (see RESULTS). Therefore quantal events after short PF
tetanus were likely to be caused by the initial activation of
presynaptic KA receptors by Glu released by PCs, i.e., the
initial phase of the KA-dependent suppression of PF-EPSCs,
rather than due to the subsequent prolonged ryanodine-sensi-
tive calcium-induced calcium release seen here. One plausible
hypothesis is that the initial activation of presynaptic KA
receptors after short PF tetanus was insufficient to trigger such
calcium-induced calcium release. Further studies will be re-
quired to determine physiological conditions in which fully
developed endocannabinoid-independent suppression of PF-
EPSCs can be induced.

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