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

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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 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-β-benzylxoxaspartate (t-TBOA) and blocked by the desensitizing kainate (KA) receptors agonist SYM 2031, by nonsaturating concentrations of 6-cyano-7-nitroquinoxaline-2-3-dione (CNQX) 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. 2001) 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 this signaling (Brown et al. 2003; Galante and Diana 2004; Maejima et al. 2001), as was previously shown for depolarization-induced suppression of inhibition (DSI) (Diana et al. 2002; Glitsch et al. 1996, 2000; Kreitzer and Regehr 2001a; Llano et al. 1991a; Ohno-Shosaku et al. 2001; Pilet and Alger 1992, 1994; Vincent et al. 1992; Wang and Zucker 2001; Wilson and Nicoll 2001; Wilson et al. 2001) and for depolarization-induced suppression of excitation (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 on the nature of retrograde messengers involved in agonist-induced suppression of excitation at PF-PC synapses might 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 C57BL/6 and GluR6−/− (on a hybrid 129Sv × C57BL/6 background) mice, as well as on juvenile (10–12 days old) C57BL/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.

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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, d-threo-β-benzyloxyaspartate (d-TBOA), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), GYKI 52466 hydrochloride (GYKI), d-2-amino-5-phosphopentanoic acid (d-APV), (2S,4R)-4-methylglutamate (SYM 2081), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), CGP55845-A [(piperidine-1-yl)-5-(4-iodomethyl)-1-(2,4dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM-251)], and ryanodine were purchased from Tocris (Ilkirch, France). The CBl cannabinoid receptor antagonist SR141716A [N-(piperidine-1-yl)-5-(4chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazol-3-carboxamide hydrochloride] was provided by Sanofi-Recherche (Montpellier, France). N,N,N-Trimethyl-L-argininemethyl ester (L-NAMe) and guanosine 5’-[β-thi]diphosphate (GDP-β-S) was purchased from Sigma Aldrich. Stock solutions of drugs (dissolved in water or DMSO depending on manufacturer recommendations) were added to the oxygenated saline solution at the desired concentration.

Electrophysiology

Whole cell patch-clamp recordings were performed from PC somas, using an Axopatch-200A amplifier (Axon Instruments). Stimulating electrodes consisted in saline filled monopolar electrodes. PF stimulations were performed at 0.33 Hz, except for studies on unitary quantal events and on DSE, where stimulation rates were 0.1 and 0.5 Hz, respectively. Patch pipettes (2–4 MΩ) were filled with a solution containing (in mM) 140 K-gluconate, 6 KCl, 10 HEPES, 0.75 EGTA, 1 MgCl₂, 4 Na₂ATP, and 0.4 Na-GTP, pH 7.35 with KOH; 300 mOsm. In experiments on DSE, patch pipettes (2–4 MΩ) were filled with a solution containing (in mM) 140 Cs-gluconate, 6 KCl, 10 HEPES, 0.2 EGTA, 1 MgCl₂, 4 Na₂ATP, and 0.4 Na-GTP (pH and osmolarity adjusted accordingly). The components of internal solutions were purchased from Sigma Aldrich.

In the cells retained for analysis, access resistance (usually 5–10 MΩ) was partially compensated (50–70%), according to the procedure described by Llano et al. (1991b). Cells were held at a membrane potential of ~70 mV, and PF-EPSCs were subjected to 10-mV hyperpolarizing voltage steps that allowed monitoring of the passive electrical properties of the recorded cell throughout the experiment (Llano et al. 1991b).

For paired-pulse facilitation (PPF) experiments (Altieri and Regehr 1996; McNaughton 1982; Schultz et al. 1994), PF stimulations of the same intensity were applied to the cell with an interstimulus interval of 30 ms, and the ratio of the amplitude of the second PF-EPSC over the first one was calculated on-line. Mean PPF values were obtained by averaging PPFs in individual traces for each cell studied in a given condition. Although this conventional method can produce spurious results (Kim and Alger 2001), no use was made of the alternative method proposed by these authors because both methods produced very similar results in a recent study on DSE at PF-PC synapses (Crepel 2007). Because PPF increases associated to agonist-induced suppression of PF-EPSCs could be small in certain experimental conditions (see RESULTS) and obscured by variability of basal PPF across cells, mean PPFs were further normalized in these experiments by determining for each cell mean normalized PPF = 100 × (mean PPF/PPFi), where PPFi was the individual PPF value of the last trace preceding the depolarizing step. The contribution of presynaptic factors in the variation of synaptic responses was also examined by using the coefficient of variation (CV) (Kullmann 1994; Martin 1966), where CV is given by: CV² = (s/M)², in which s is the SD of the amplitude distribution of EPSCs corrected for the background noise, and M is the mean amplitude of EPSCs during the same epoch. In these experiments, CV were calculated on sets of 40–60 stable EPSCs according to the procedure previously described (Blond et al. 1997).

Fluorometry

Parallel fibers in coronal slices from nearly mature and juvenile rats (see RESULTS) were loaded with focal application of 100 μM of the low-affinity calcium sensitive dye Fluo-4FF-AM (Molecular Probes) as previously described (Levenes et al. 2001). After loading for ≥45 min, fluorescent signals from labeled PFs were recorded in a 20 × 50-μm window placed above the molecular layer, 500–800 μm away from the loading site and 100 μm above PC layer. The epifluorescence excitation light at 485 ± 22 nm was gated with an electromechanical shutter (Uniblitz, Rochester, NY), and the emitted light was collected by a photometer through a barrier filter at 530 ± 30 nm. The rather selective and weakly desensitizing KA receptor agonist domoate (Lerma et al. 1993) was applied for 1 min and at a concentration of 20 μM by local superfusion through a theta-tube (rate of 0.5 ml/min) placed ~50 μm above the surface of the slice, at the level of the molecular layer and parallel to labeled PFs. With such a protocol that minimized possible movements of the slice when switching superfusion through the theta-tube from standard saline solution to that with domoate included, this compound was unlikely to diffuse in significant concentration to nearby granule cells, whereas it was probably still at near saturating concentrations for presynaptic KA receptors (Renard et al. 1995), and this, even after partial dilution during superfusion of the tissue. In these experiments, the 1-min superfusion duration was chosen because, in pilot experiments, it was the shortest time that gave rise to reliable presynaptic calcium signals. Fluorescence signals corrected for dye bleaching and background fluorescence were expressed as relative fluorescence changes ΔF/F, where F was the baseline fluorescence intensity and ΔF was the change induced by domoate.

In sagittal slices, epifluorescence microscopy was also used to detect variations in intracellular free calcium concentration changes from an area of 90 × 90 μm centered on dendrites of recorded PCs, as previously described (Crepel 2007). In these experiments, the low affinity and impermeant calcium indicator Fluo-4FF-AM (100 μM, Molecular Probes) was added to the same Cs-gluconate–based solution as used in experiments on DSE. The recording session started 30–45 min after whole cell break in, to allow diffusion of the dye to the dendrites. Fluorescence data corrected for dye bleaching and background fluorescence were again expressed as changes in ΔF/F, where F was the baseline fluorescence intensity, and ΔF was the fluorescence change induced by depolarization of PC soma from ~70 to 0 mV for 1 s.

In all cases, pre- and postsynaptic calcium signals were recorded while superfusing the slices with a cocktail of GABA_A, GABA_B and adenosine A1 receptor antagonists, i.e., bicuculline methochloride (10 μM), CGP55845 (300 nM), and DPCPX (100 nM), respectively. Fluorometric measurements were analyzed on- and off-line using the Acquis1 computer program (Biologic).

Statistical significance was assessed by paired or unpaired t-test, as appropriate, with P < 0.05 (2-tailed) considered significant. All error values given are mean ± SE.

RESULTS

Agonist-induced suppression of PF-EPSCs depends entirely on retrograde release of endocannabinoids in juvenile rodents but only partly in nearly mature ones

In 10- to 12-day-old mice, as shown in the previous study by Maejima et al. (2001), 5-min bath application of the selective mGluR1 and mGluR5 agonist DHPG, at an apparent saturating concentration of 100 μM (Canepari et al. 2004; Pin
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 (Gatley 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, B and C). 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. 2, A and B1). 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. 2B2). 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).1 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.

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1 The online version of this article contains supplemental data.
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 μM SR141716-A and as expected for a Glu uptake inhibitor, application of 100 μM 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 ± 7.97% (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 μM DHPG decreased the mean amplitude of PF-EPSCs that amounted to 39.78 ± 4.63% of control amplitude before DHPG application (Fig. 3, 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 ± 0.17 in SR141716-A + D-TBOA containing medium to 1.77 ± 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 with the view that DHPG-induced 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 ± 0.21 and 1.90 ± 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 ± 0.75 and 10.90 ± 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, perfusing nearly mature slices for ≥30 min with 1 μM SR141716-A only partly inhibited the DHPG-induced suppression of PF-EPSCs. Indeed, mean amplitude decrease was only 22.11 ± 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 μM 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 μM did not further antagonize this inhibition of PF-EPSCs. Indeed, in the presence of 2 μM AM-251, the mean decrease in PF-EPSC amplitude in the presence of 100 μM DHPG was 26 ± 3.75% (n = 6; data not shown), a value very similar to that obtained in the presence of 1 μM 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 μM bath application of the selective CB1 receptor agonist WIN55,212–2 (Devane et al. 1988) on PF-EPSCs (n = 3 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 ± 0.05 in control conditions to 1.51 ± 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 ± 0.21 and 1.90 ± 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 ± 0.75 and 10.90 ± 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).
independentsuppression of PF-EPSCs induced by bath application of 100 μM DHPG totally inhibited the late phase of the endocannabinoid-independent suppression of PF-EPSCs in nearly mature rats. 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

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; Epsztejn 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.

FIG. 3. Effect of D-threo-β-hydroxyaspartate (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 D-TBOA were normalized with respect to values immediately before DHPG application. B2: plots of mean 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,4R)-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 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 + 100 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_B receptors (Dittman and Regger 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 (Baudet 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 C, 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 (Honore 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 GluR6−/− 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 GYKI, a noncompetitive and more selective 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 decrease in mean 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 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).

GDH-β5 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
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 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-S, a clear-cut endocannabinoid-independent suppression of PF-EPSCs and associated PPF increase were still induced by bath application of 100 μM DHPG (Fig. 7, A and B).

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 itself, 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 unbalance after large depolarizing steps in postsynaptic cells (Crepel 2007).

Altogether, the marked effects of ryanodine on agonist-induced suppression of PF-EPSCs and on DSE at PF-PC synapses suggests that, as for DPI, their prolonged duration involves long-lasting calcium release from presynaptic ryanodine-sensitive calcium stores, after an initial and more short-lived activation of presynaptic KA receptors. Moreover, the fact that ryanodine also partly inhibited the early phase of the CB1 receptor–independent suppression of PF-EPSCs by DHPG, i.e., the portion of the response that was resistant to bath application of SYM 2081 (cf. Figs. 4A and 8B1), suggests in turn that ryanodine also inhibits the presynaptic GABA<sub>B</sub><sup>1</sup> and NO-dependent components of agonist-induced suppression of PF-EPSCs.

In these experiments, inhibition of the Glu-dependent component of the agonist-induced suppression of PF-EPSCs by ryanodine might also result from an inhibition of calcium release from postsynaptic ryanodine-sensitive calcium stores (Carter et al. 2002; Isokawa and Alger 2006) that would in turn inhibit retrograde release of Glu from PCs. To compensate, at least partly, for these postsynaptic effects, we used 2-s rather than 1-s 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

![Figure 8](http://jn.physiology.org/)

**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 during 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.
in PFs by focal application (see METHODS) of the rather selective and weakly desensitizing KA receptor agonist domoate (Lerma et al. 1993) on the other hand. The latter protocol was directly derived from that used by Duguid and Smart (2004) to show involvement of presynaptic calcium-induced calcium release in DPI.

**Sensitivity to ryanodine of pre- and postsynaptic calcium signaling involved in the CB1 receptor–independent component of DSE in nearly mature rats**

PFs loaded with Fluo-4FF-AM were subjected to focal superfusion of 20 μM domoate for 1 min. This elicited a transient increase in the cytosolic calcium signal in these fibers that peaked shortly after the end of domoate application and relaxed slowly thereafter in <30 min (n = 6; Fig. 9, A1 and A2). Ryanodine (100 μM) present in the bath for ≥30 min markedly reduced the duration of domoate-induced calcium transients, whereas the peak calcium transient was only marginally affected (n = 5; Fig. 9, A1 and A2). Indeed, peak ΔF/F in control conditions and in the presence of ryanodine were 3.58 ± 0.10 and 3.42 ± 0.94%, respectively; these values are not significantly different. To further quantify differences in calcium transients recorded in control conditions and in the presence of ryanodine, we determined for each cell θ = ∑ (ΔF/F) for each point of ΔF/F plots between the beginning of domoate application and 30 min thereafter. We averaged these values for all cells recorded in a given condition (see Crepel 2007). Mean θ was significantly lower in the presence of ryanodine, because it amounted to only 0.61 ± 0.14 compared with 1.52 ± 0.30 in its absence (P < 0.05). These results 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 ΔF/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 ΔF/F was now only 7.77 ± 1.63% (%). Differences between control and ryanodine results were further quantified by determining again, for each cell, mean θ values calculated from the start of the depolarizing step until 4 s after, i.e., the period of time during which ΔF/F plots in control conditions and in the presence of ryanodine appeared clearly different (Fig. 9B2). The mean θ value was significantly lower (P < 0.05) with 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-induced suppression 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-induced 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 t-TBOA (100 μM) did not unmask any sizeable Glu-dependent component of agonist-induced 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 counterbalanced 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 t-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 ΔF/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 (ΔF/F = 4.74 ± 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 Δ 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 super fused 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 probably present and functional 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. 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 probably present and functional 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.
In 12-day-old rats, the mean $\bar{\theta}$ values of calcium transients elicited in the presence of D-TBOA and D-APV or in the presence of D-TBOA and ryanodine were still significantly ($P < 0.05$) higher (0.96 ± 0.18 and 0.96 ± 0.30, respectively) than in control medium (0.10 ± 0.02; Fig. 10B2). It is therefore likely that an additional mechanism participates to calcium transients induced by domoate in PFs in the presence of D-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 basal calcium concentration within PFs and facilitate induction of calcium-induced calcium release on activation of presynaptic KA receptors. If this was so, prolonged calcium transients induced by domoate in the presence of D-TBOA and D-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 D-TBOA and 50 $\mu$M D-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 D-TBOA and D-APV or in the presence of D-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-BS are consistent with the view that Glu mainly originates from neighboring cells and/or not fully developed calcium release from ryanodine-sensitive calcium stores. Finally, results in nearly mature rats (Crepel 2007), Glu is likely to depend partly on retrograde release of glutamate and activation of presynaptic KA receptors. Moreover, and as mentioned above, results in nearly mature glutamate-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-, GABA$\gamma$-, 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 imbalance 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 potentiating effect of DHPG on PF-EPSCs 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 dendritic 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 SYM 2081 as well as by competitive AMPA/KA receptor antagonist CNQX (Honore 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-BS 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_{B} 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 PPF 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 PFF stimulations, before reaching a quasi steady state (see Fig. 5A). Along the same line, increasing the frequency of PFF 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 \( r = \text{mean basal PPF increment/mean EPSC amplitude decrease during superfusion of the slices with GYKI} \) was only 0.29 compared with 0.97 and 0.76, respectively, for the ratio \( R = \text{mean PPF increment/mean EPSC amplitude decrease during DHPG application in the presence of SR141716-A + GYKI and in the presence of 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 participation 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
ryanthione also markedly reduced basal PPF values

cium increase responsible for basal PPF at PF-PC synapses

dine-sensitive calcium stores participate in the residual cal-

input. However, in these experiments, this enhanced discharge

erm of local communication among PCs sharing the same PF

ances of animals under study explain the apparent contradic-

ing the duration of the KA-dependent component of

B1 (Crepel 2007).

In addition, these results also suggest that presynaptic ryan-
odine-sensitive calcium stores participate in the residual cal-
cue increase responsible for basal PF at PF-PC synapses

dine-sensitive calcium stores in these experiments was about twice that of inhibitory synap-
to retrograde release of Glu from PCs. However, this contrib-
time is longer than duration of the KA-sensitive component of

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

cules 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-
ences 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-

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.

A C K N O W L E D G M E N T S

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R E F E R E N C E S

Ali AB, Rossier J, Staiger JF, Audinat E. Kainate receptors regulate unitary
IPSCs elicited in pyramidal cells by fast-spiking interneurons in the neo-

Angulo MC, Kozlov AS, Charpak S, Audinat E. Glutamate released from
glia cells synchronizes neuronal activity in the hippocampus. J Neurosci 24:

Allturi P, Regehr WG. Determinants of the time course of facilitation at the

Barbour B, Keller BU, Llano I, Marty A. Prolonged presence of glutamate
during excitatory synaptic transmission to cerebellar Purkinje cells. Neuron

Batchelor AM, Madge DJ, Garthwaite J. Synaptic activation of metabo-
tropic glutamate receptors in the parallel fibre-Purkinje cell pathway in rat

Baude A, Nusser Z, Roberts JDB, Mulvihill E, McIlhinney RAJ, Somogyi
P. The metabotropic glutamate receptor (mGluR1a) is concentrated at
perisynaptic membrane of neuronal subpopulations as detected by immuno-

Berman FW, Murray TF. Domoic acid neurotoxicity in cultured cerebellar
granule neurons is mediated predominantly by NMDA receptors that are
activated as a consequence of excitatory amino acid acid release. J Neurochem

Blond O, Daniel H, Jaillard D, Crepel F. Pre- and postsynaptic effects of
nitric oxide at synapses between parallel fibers and Purkinje cells; involve-

Bredt S, Hwang PM, Snyder SH. Localization of nitric oxide synthase

Brenovitz SD, Regehr WG. Calcium dependence of retrograde inhibition by
endocannabinoids at synapses onto Purkinje cells. J Neurosci 23: 6373–

Brown SP, Brenovitz SD, Regehr WG. Brief pre-synaptic bursts evoke
synapse-specific retrograde inhibition mediated by endogenous cannabi-

Bureau I, Bischoff S, Heinemann SF, Mulle C. Kainate receptor-mediated
responses in the CA1 field of wild-type and GluR6-deficient mice. J Neu-

Canepari M, Auger C, Ogden D. Calcium ion permeability and single-
channel properties of the metabotropic slow EPSC of rat Purkinje neurons.

Carter AG, Vogt KE, Foster KA, Regehr WG. Assessing the role of
calcium-induced calcium release in short-term presynaptic plasticity at

Cassado M, Dieudonne S, Ascher P. Pre-synaptic N-methyl-D-aspartate
receptors at the parallel fiber-purkinje cell synapse. Proc Natl Acad Sci USA

Cho K, Francis JC, Hibbec H, Dev K, Brown MW, Henley JM, Bashir ZI.
Regulation of kainate receptors by protein kinase C and metabotropic

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