Selective Inhibition of Spontaneous But Not Ca\textsuperscript{2+}-Dependent Release Machinery by Presynaptic Group II mGluRs in Rat Cerebellar Slices

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Glitsch, Maike. Selective inhibition of spontaneous but not Ca\textsuperscript{2+}-dependent release machinery by presynaptic group II mGluRs in rat cerebellar slices. J Neurophysiol 96: 86–96, 2006. First published April 12, 2006; doi:10.1152/jn.01282.2005. Two main forms of neurotransmitter release are known: action potential-evoked and spontaneous release. Action potential-evoked release depends on Ca\textsuperscript{2+} entry through voltage-gated Ca\textsuperscript{2+} channels, whereas spontaneous release is thought to be Ca\textsuperscript{2+}-independent. Generally, spontaneous and action potential-evoked release are believed to use the same release machinery to release neurotransmitter. This study shows, using the whole-cell patch-clamp technique in rat cerebellar slices, that at the interneuron-Purkinje cell synapse activation of presynaptic group II metabotropic glutamate receptors suppresses spontaneous GABA release through a mechanism independent of voltage-gated Ca\textsuperscript{2+} channels, store-operated Ca\textsuperscript{2+} channels, and Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores, suggesting that the metabotropic receptors target the release machinery directly. Voltage gated Ca\textsuperscript{2+} channel-independent release following increased presynaptic cAMP production is similarly inhibited by these metabotropic receptors. In contrast, both voltage-gated Ca\textsuperscript{2+} channel-dependent and presynaptic N-methyl-d-aspartate receptor-dependent GABA release were unaffected by activation of group II metabotropic glutamate receptors. Hence, the mechanisms underlying spontaneous and Ca\textsuperscript{2+}-dependent GABA release are distinct in that only the former is blocked by group II metabotropic glutamate receptors. Thus, the same neurotransmitter, glutamate, can activate or inhibit neurotransmitter release by selecting different receptors that target different release machineries.

INTRODUCTION

Neurotransmitter release is generally taken as an example of regulated exocytosis: on an appropriate stimulus (usually the action potential, AP), a neuron releases neurotransmitter onto other neurons. The function of the AP is to depolarize the terminal, thereby opening voltage-gated Ca\textsuperscript{2+} channels (VGCCs). Ca\textsuperscript{2+} influx through these channels then triggers the fusion of the neurotransmitter-filled vesicle with the presynaptic membrane, thereby releasing its content into the synaptic cleft. However, neurotransmitter release can also occur in the absence of APs. This form of exocytosis is called spontaneous release because of the apparent lack of any obvious stimulus that might trigger this release. Generally, spontaneous release occurs at a much slower rate than AP-dependent release.

It is unclear why these two neurotransmitter release pathways exist in parallel, but it seems that the AP-dependent pathway is used primarily for information transfer whereas the spontaneous pathway may also serve such diverse functions as synapse structure maintenance (McKinney et al. 1999), regulation of dendritic protein synthesis (Sutton et al. 2004), shaping of nerve cell firing (Carter and Regehr 2002; Cohen and Miles 2000) and contribution to synaptic plasticity (Jensen et al. 1999; Kombian et al. 2000).

It has been shown for some synapses that spontaneous and AP-induced neurotransmitter release use the same vesicle pool and release machinery (Prange and Murphy 1999; Rosenmund and Stevens 1996; Scanziani et al. 1995; Wu and Saggau 1997). At other synapses, however, spontaneous and evoked release employ different release machineries and/or vesicle pools (Calakos et al. 2004; Deitcher et al. 1998; Humeau et al. 2000; Koenig and Ikeda 1999; Maruyama et al. 2001; Sara et al. 2005).

AP-dependent and spontaneous neurotransmitter release are both subject to modulation by G-protein-coupled receptors, and in many systems, activation of these receptors leads to a decrease in presynaptic neurotransmitter release (reviewed in Miller 1998; Offermanns 2003; Wu and Saggau 1997).

In principle, G-protein-coupled receptors can reduce neurotransmitter release by inhibiting VGCCs or by opening inwardly rectifying K\textsuperscript{+} channels; this prevents or reduces terminal depolarization. Alternatively, activation of G-protein-coupled receptors could modulate Ca\textsuperscript{2+} release from intracellular stores or even directly inhibit the neurotransmitter release machinery itself (Blackmer et al. 2001, 2005; Gerachshenko et al. 2005; Miller 1998; Offermanns 2003; Stevens 2004; Sullivan 2005).

At the interneuron-Purkinje cell synapse in rat cerebellar slices, it has been shown that activation of presynaptic metabotropic glutamate receptors (mGluRs) decreases both spontaneous and AP-dependent GABA release (Glitsch et al. 1996; Llano and Marty 1995). It is not clear whether activation of mGluRs blocks both forms of exocytosis through similar or distinct intracellular signaling pathways.

This study demonstrates that spontaneous and depolarization-induced GABA release are differentially regulated by presynaptic group II mGluRs in that only spontaneous GABA release is inhibited. Hence group II mGluRs selectively target the vesicles/release machinery responsible for spontaneous release.

METHODS

Tissue preparation

Sagitital cerebellar slices were obtained from Sprague Dawley rats aged 13–15 days as previously described (Glitsch et al. 1996; Llano et al. 1991). Briefly, rats were decapitated after cervical dislocation, and...
the cerebellar vermis was dissected out. The vermis was cooled in ice-cold bicarbonate-buffered saline (BBS, composition see following text) for 2–4 min and then glued to the stage of a vibratome. Slices of 200 μm thickness were cut and incubated in oxygenated BBS at room temperature for 30 min before their use in experiments.

**Electrophysiology**

Tight-seal whole cell recordings were obtained from Purkinje cells, as described in Glitsch et al. (1996) and Llano et al. (1991). A ×63 water-immersion objective was used for visualization of the slices during experiments. Experiments were performed using an EPC9/2 patch-clamp amplifier (Heka Electronics, Lambrecht, German). Patch pipettes had resistances of 2–3 MΩ with the standard internal and extracellular solutions used (composition see following text). If series resistance >10 MΩ or the holding current at −60 mV holding potential exceeded −500 pA, experiments were abandoned. Capacitive currents were cancelled and series resistance was compensated by between 50 and 80%, as described in Llano et al. (1991).

Experiments were performed at room temperature and slices were continuously perfused with oxygenated BBS at a rate of 2 ml/min. Drugs were applied to the slice by either dissolving them directly in BBS or by dissolving prepared stock solutions into BBS to the desired concentration. BBS was applied via the bath perfusion system at a high perfusion speed (>5 ml/min) for ≤1 min. To study the effect of a drug on synaptic transmission, synaptic currents were recorded for 3 min under control conditions, starting 2 min after establishing the whole cell configuration. Then the drug was washed in and recordings were resumed for 3 min under test conditions. In some cases, a third recording was made. For some experiments, slices were preincubated in the presence of the drugs to ensure maximal drug exposure.

Only one cell per slice was used for each experiment to avoid contamination of the results by potential long-term side effects of the drugs used.

**Solutions and drugs**

The standard external solution BBS contained (in mM) 125 NaCl, 2.5 KCl, 26 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, and 10 glucose (pH 7.4 when oxygenated with carbogen). For experiments with elevated extracellular K+ concentration, 12.5 mM NaCl was replaced by 12.5 mM KCl.

The intracellular solution contained (in mM) 150 CsCl, 10 HEPES, 1 EGTA, 0.1 CaCl2, 4 Na-ATP, 0.4 Na-GTP, and 4.6 MgCl2 (pH adjusted to 7.3 with CsOH).

(2S,2R,3R)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV), tetrodotoxin (TTX), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[fl]quinoxaline-7-sulfonamide (NBQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; disodium salt), N-methyl-d-aspartic acid (NMDA), and forskolin were obtained from Tocris Cookson (Bristol, UK). CdCl2, nifedipine, ranodine, and thapsigargin were obtained from Sigma Aldrich (Poole, UK). Apart from nifedipine, ranodine, forskolin, 2-aminoethoxydiphosphorylborane (2-APB) and thapsigargin, which were dissolved in DMSO, all drugs were either dissolved in water or BBS. Control experiments for drugs dissolved in DMSO were carried out in the same final concentration of DMSO to ensure that DMSO per se did not have any effects on synaptic transmission. NBQX was dissolved in equimolar NaOH. Drug stocks were aliquoted and frozen at −20°C. All experiments were carried out in the presence of 0.25 μM TTX and 10 μM NBQX or CNQX; the DCG-IV concentration used was always 5 μM. The TTX concentration used was high enough to completely suppress any APs both pre- and postsynaptically, even in the presence of elevated external potassium (see also Glitsch and Marty 1999).

**Analysis**

Synaptic currents were analyzed using an event-detection routing kindly provided by Dr. P. Vincent. Each event above the selected detection threshold was visually confirmed to be a synaptic current (Vincent and Marty 1993).

Data are given as means ± SE for n numbers of cells. Student t-test was carried out using InStat 2.03 for Macintosh and SPSS 12.0 for Windows, and paired tests were used where appropriate. Results were considered significant with P ≤ 0.05.

Data were normalized by calculating the average control value [average control amplitude or average control frequency of miniature inhibitory postsynaptic currents (mIPSCs)]. Control and test values were then divided by this average control value and converted to % normalized amplitude or % normalized number of events.

**RESULTS**

It was previously shown that activation of presynaptic group II mGluRs reduced GABA release from presynaptic interneurons in rat cerebellar slices in the presence of TTX (Glitsch et al. 1996; Llano and Marty 1995). Figure 1A shows that activation of type II mGluRs with the specific group II mGluR agonist DCG-IV (5 μM, a maximally effective dose) (Glitsch et al. 1996) leads to a reduction in mIPSC frequency. Aggregate data from 16 Purkinje cells is summarized in Fig. 1, A and B. Application of 5 μM DCG-IV led to a decrease in mIPSC frequency by 63.4 ± 3.6%, which was highly significant (P < 0.0001; Fig. 1B), whereas mIPSC amplitude was unaffected (P = 0.4876; Fig. 1C).

Although this pattern is similar to the findings reported previously (Glitsch et al. 1996), there are some minor differences. In the previous study, there was a small but significant reduction in mIPSC amplitude (15 ± 4% reduction) after
DCG-IV application that was not observed in the present study. Also, the effect of DCG-IV on mIPSC frequency was less pronounced in the previous study (45 ± 4.5% reduction) compared with that here (63.4 ± 3.6% reduction). Moreover, in the previous study 17% of Purkinje cells showed no decrease in mIPSC frequency after DCG-IV applications, whereas here all Purkinje cells displayed a decrease in mIPSC frequency (n = 45). The reasons for these discrepancies are unclear but are likely to reflect a difference in rat ages and/or strains used (rat ages here: P13-15, previous study: P11-12; rat strain here: Sprague Dawley, previous study: Wistar).

Spontaneous neurotransmitter release could in principle be the result of spontaneous opening of voltage-gated Ca²⁺ channels. Experiments were designed to test whether activation of presynaptic group II mGluRs reduced spontaneous GABA release by inhibiting VGCCs (recently reviewed in Elmslie 2003).

Figure 2A shows that in the presence of 200 μM Cd²⁺, mIPSCs can still be recorded. There was a significant reduction in mIPSC frequency (30.5 ± 14.8%; P = 0.004; Fig. 2B), which would suggest that at least some mIPSCs result from the spontaneous opening of VGCCs. However, these results have to be seen in conjunction with the corresponding, significant reduction in mIPSC amplitude after Cd²⁺ application (16.4 ± 4.3%; P = 0.013; Fig. 2C; n = 9). It is well documented in the literature that currents through GABA_A receptors are reduced in the presence of Cd²⁺ (Casagrande et al. 2003; Celentano et al. 1991; Smart and Constanti 1990). The small reduction in mIPSC frequency observed after addition of Cd²⁺ (Fig. 2B) can therefore most easily be explained by the small reduction in mIPSC amplitude that occurs in the presence of Cd²⁺ (Fig. 2C). This will result in a reduction in mIPSC frequency because more synaptic events will fall under the detection threshold, thereby effectively reducing the number of events that are detected.

Supplementary Fig. 1 shows a correlation between average mIPSC amplitude under control conditions and percent reduction in mIPSC frequency after Cd²⁺ application. The smaller the initial average mIPSC amplitude (and hence the more small mIPSCs contribute to the small average amplitude), the bigger the relative reduction in mIPSC frequency, whereas the bigger the initial average mIPSC amplitude (and the less small amplitude mIPSCs contribute to it), the smaller the mIPSC frequency reduction after Cd²⁺ application. These results suggest that it is indeed the reduction in mIPSC amplitude that gives rise to an apparent decrease in mIPSC frequency after Cd²⁺ application because cells with a small initial average mIPSC amplitude are more affected than those with a large average mIPSC amplitude.

Importantly, even in the presence of Cd²⁺, addition of 5 μM DCG-IV produced a highly significant reduction in mIPSC frequency (Fig. 2A, aggregate data summarized in B; P = 0.001). The extent of reduction observed in the presence of Cd²⁺ was similar to that under control conditions after application of DCG-IV (when comparing frequency of mIPSCs in presence of Cd²⁺ and frequency of mIPSC in presence of Cd²⁺ and DCG-IV; P = 0.3338).

These findings are in good agreement with other studies that have shown that neurotransmitter release in the presence of TTX is independent of presynaptic VGCCs (Bouron 2001; Harvey and Stephens 2004; Miller 1998; Scanziani et al. 1992, 1995; Thompson et al. 1993).

Generally, only P/Q-, N-, and R-type voltage-gated Ca²⁺ channels are believed to play a role in neurotransmitter release (reviewed in Catterall 2000; Stevens 2004), and Cd²⁺ is known to fully block all these channels at concentrations similar to or lower than that used here. However, to ensure that there was no unexpected role for T-type Ca²⁺ channels in spontaneous neurotransmitter release, experiments were repeated in 200 μM Ni²⁺ which blocks T-type Ca²⁺ channels (as well as R- and to some extent P-type Ca²⁺ channels).
In the presence of 200 μM Ni²⁺, DCG-IV reduced presynaptic GABA release by 55.6 ± 11.4% (P = 0.028, n = 4; Fig. 2D). Even in the combined presence of 200 μM Ni²⁺ and 200 μM Cd²⁺, DCG-IV still suppressed mIPSC frequency by 60.3 ± 13% (P = 0.009, n = 4; Fig. 2E). As was the case with Cd²⁺, Ni²⁺ produced a small reduction in mIPSC frequency and amplitude (data not shown for effect of Ni²⁺ on amplitude).

The reduction in mIPSC frequency after DCG-IV application in the presence of both Ni²⁺ and Cd²⁺ was not significantly different to the reduction induced by DCG-IV under control conditions (P = 0.745; Figs. 1B and 2E), suggesting that VGCCs are not involved in the group II mGluR-mediated reduction in presynaptic GABA release. Moreover, these results show that spontaneous GABA release from presynaptic interneurons is largely independent of presynaptic VGCC activity.

In nonexcitable cells, Ca²⁺ entry through store-operated channels can drive secretory events (recently reviewed by Parekh and Putney 2005). However, the store-operated channel blocker 2-APB (20 μM) had no significant inhibitory effect on spontaneous release (13.2 ± 11% reduction in mIPSC frequency with no effect on mIPSC amplitude; the reduction inaptic GABA release by 55.6 (Prange and Murphy 1999; Rosenmund and Stevens 1996; Scanziani et al. 1995; Wu and Saggau 1997).

There is evidence that Ca²⁺ release from presynaptic Ca²⁺ stores contributes to GABA release both at the interneuron–Purkinje cell synapse (Bardo et al. 2002; Galante and Marty 2003; Llano et al. 2000) and at other synapses (for review, see Bouron 2001; Bouchard et al. 2003). Although group II mGluR activation can reduce spontaneous GABA release in the absence of functional VGCC, it is possible that they modulate Ca²⁺ release from stores.

Slices were preincubated in 20 μM ryanodine (a concentration previously shown to release Ca²⁺ from intracellular stores) (Llano et al. 1994, 2000), 2 μM thapsigargin (a SERCA pump blocker that prevents refilling of intracellular stores), and 200 μM Cd²⁺ (on average 47 ± 4.2 min incubation; n = 9). Under these conditions, Ca²⁺ influx through VGCCs should be blocked by Cd²⁺, and intracellular Ca²⁺ stores should be depleted by the combination of ryanodine and thapsigargin.

As can be seen in Fig. 3A, even in the presence of these drugs, there was still a clear and significant reduction in mIPSC frequency after application of DCG-IV. Aggregate data are summarized in Fig. 3C (left). DCG-IV significantly reduced mIPSC frequency after exposure to ryanodine/thapsigargin/Cd²⁺, and the extent of this reduction was similar to that seen in the presence of Cd²⁺ but where intracellular Ca²⁺ stores were intact (Fig. 3, B and C, right; n = 9).

It cannot be ruled out that a presynaptic ryanodine- and thapsigargin-insensitive Ca²⁺ store is involved in regulating spontaneous GABA release and that this store is targeted by group II mGluRs. Although such a store has been proposed for certain nonexcitable cells (Genazzani and Galione 1996), its existence remains controversial (Gerasimenko and Gerasimenko 2004; Gerasimenko et al. 2003).

These data demonstrate that spontaneous GABA release can still be blocked by activation of group II mGluRs even in the absence of functional VGCC and functional ryanodine- and thapsigargin-sensitive Ca²⁺ stores.

The preceding results clearly demonstrate that activation of presynaptic group II mGluRs by DCG-IV reduces spontaneous GABA release through a mechanism that is independent of VGCC, store-operated channels, and intracellular Ca²⁺ stores, suggesting that group II mGluRs target directly the release machinery (Blackmer et al. 2001, 2005; Cartmell and Schoepf 2000; Gerachshenko et al. 2005; Offermanns 2003; Sullivan 2005). In some mammalian synapses, spontaneous and depolarization-induced neurotransmitter release utilize the same release machinery and the same pool of synaptic vesicles (Prange and Murphy 1999; Rosenmund and Stevens 1996; Scanziani et al. 1995; Wu and Saggau 1997).
If this is also true for the interneuron–Purkinje cell synapse, then one would expect activation of presynaptic group II mGluRs, which reduces spontaneous release, to also impair depolarization-induced transmitter release. To test this, terminals were depolarized directly by raising extracellular K\(^+\) concentration sixfold (external K\(^+\) raised by 12.5 mM to 15 mM; subsequently referred to as elevated K\(^+\)).

Raising external K\(^+\) to 15 mM depolarizes the presynaptic terminals, thereby activating VGCCs. This results in a prominent increase in presynaptic GABA release (Fig. 4A). The IPSCs recorded in the presence of TTX and elevated K\(^+\) are a mixture of both VGCC-mediated IPSCs and mIPSCs and will henceforth be called tIPSCs (t standing for total). If both depolarization-dependent and spontaneous GABA release link to the same pool of vesicles/release machinery, then addition of DCG-IV should lead to a pronounced reduction in depolarization-mediated GABA release.

Figure 4A shows that DCG-IV, at a concentration and exposure time that substantially reduced spontaneous release, failed to inhibit increased GABA release following presynaptic depolarization with 15 mM K\(^+\). Whereas elevated K\(^+\) significantly increased tIPSC frequency (Fig. 4C; \(P = 0.0075; n = 9\)), subsequent addition of DCG-IV had no significant effect (Fig. 4C).

In the experiments of Fig. 4A, cells were first exposed to elevated K\(^+\), and after synaptic activity had been recorded, DCG-IV was added (still in elevated K\(^+\)). It is possible that prolonged exposure to elevated K\(^+\) leads to a time-dependent increase in tIPSC frequency, and this could conceivably mask potential inhibitory effects of DCG-IV. To test this, tIPSCs were monitored in the continuous presence of elevated K\(^+\) (without adding DCG-IV).

As shown in Fig. 4B, there is indeed a further increase in tIPSC frequency with prolonged exposure to elevated K\(^+\). Aggregate data are summarized in Fig. 4D, which compares the tIPSC frequency in control conditions, after 1-min exposure to elevated K\(^+\) (called K) and then after 6-min exposure (called pK). After 6-min exposure, tIPSC frequency increased significantly (\(P = 0.022\)) compared with tIPSC frequency after 1 min. Because of this time-dependent increase in tIPSC frequency in elevated K\(^+\), it was important to compare tIPSC frequency in elevated K\(^+\) with that in elevated K\(^+\) + DCG-IV but after equal exposure time to elevated K\(^+\).

Figure 4E compares the normalized tIPSC frequency obtained after prolonged (6 min) exposure to elevated K\(^+\) with the normalized tIPSC frequency obtained in the additional presence of DCG-IV (and exposed to elevated K\(^+\) for the same time; \(n = 9\) for each condition).

Although the frequency of tIPSC in the presence of DCG-IV was slightly reduced when compared with elevated K\(^+\) alone, this reduction was not significant (Fig. 4E; \(P = 0.1605\)). Moreover, a small reduction in tIPSC frequency is not unexpected because even in the presence of elevated K\(^+\) (which increases presynaptic GABA release about threefold), there will be a certain number of mIPSCs within this tIPSC population that will be blocked after activation of presynaptic group II mGluRs, giving rise to the small reduction of tIPSC frequency in the presence of DCG-IV.

The important finding is that the difference between extent of reduction in tIPSC frequency after addition of DCG-IV in the presence of elevated K\(^+\) and under control conditions (i.e.,
were significant (P = 0.0083). If both spontaneous and depolarization-induced GABA releases were subject to inhibition after activation of presynaptic group II mGluRs, then there should be no difference in the extent of reduction of mIPSC frequency after DCG-IV application in the presence and absence of elevated K\(^+\). The fact that the effect of DCG-IV is significantly less in the presence of elevated K\(^+\) means that the increased GABA release after activation of VGCCs in elevated K\(^+\) is not subject to group II mGluR-mediated modulation.

To confirm that elevated K\(^+\) did indeed increase tIPSC frequency via activation of presynaptic VGCCs, control experiments were carried out in which the effect of 100 and 200 \(\mu\)M Cd\(^{2+}\) on elevated K\(^+\)-induced GABA release was investigated. Results for 100 and 200 \(\mu\)M Cd\(^{2+}\) were identical and therefore pooled. On average, 15 mM K\(^+\) increased tIPSC frequency by 160.6 ± 27.5% (1-min exposure) and also increased mean tIPSC amplitude by 11.8 ± 5.3% (n = 24). Both increases were significant (P < 0.0001 and P = 0.002, respectively).

In the presence of 100–200 \(\mu\)M Cd\(^{2+}\), the elevated K\(^+\)-induced increase in tIPSC frequency was largely suppressed (reduced by 80.5 ± 21.2% without affecting mean amplitude). This finding is consistent with the idea that only high-threshold VGCCs are involved in elevated K\(^+\)-dependent presynaptic GABA release (recently reviewed in Catterall 2000; Stevens 2004). Moreover, these results show that 100 \(\mu\)M Cd\(^{2+}\) is able to suppress VGCC activity, yet at the same and higher concentrations it barely affects spontaneous release (Fig. 2). Because of the somewhat unexpected time dependence of presynaptic GABA release after exposure to elevated K\(^+\), it was considered whether there might be a role for L-type VGCCs in presynaptic GABA release. However, when experiments with elevated K\(^+\) levels were repeated in the presence of 10 \(\mu\)M nifedipine, a potent L-type Ca\(^{2+}\) channel blocker, there was no difference in the extent of increase in presynaptic GABA release between control and test (i.e., in the presence of nifedipine) conditions (control increase with 15 mM K\(^+\): 257.2 ± 67.7% (n = 6); increase with 15 mM K\(^+\) in the presence of nifedipine: 249.6 ± 53.1%; n = 5), suggesting that L-type Ca\(^{2+}\) channels are not involved in the elevated K\(^+\)-mediated increase in presynaptic GABA release.

Taken together, these results show that activation of presynaptic group II mGluRs with DCG-IV does not lead to a reduction in VGCC-mediated presynaptic GABA release.

Forskolin, a potent stimulator of cAMP production through direct activation of adenylate cyclases, leads to increases in presynaptic neurotransmitter release in a number of preparations. In cerebellum, both spontaneous and action-potential induced neurotransmitter release can be enhanced after forskolin application (Chen and Regehr 1997; Glitsch et al. 1996; Kondo and Marty 1997; Salin et al. 1996).

It was therefore of interest to see whether the forskolin-induced increase in mIPSC frequency could be blocked by activation of group II mGluRs. Figure 5A shows that application of 10 \(\mu\)M forskolin (recordings resumed after 2-min perfusion with forskolin) led to a clear increase in mIPSC frequency, and this increase was statistically significant compared with DMSO (solvent for forskolin) controls (P = 0.0181; Fig. 5D). Subsequent addition of DCG-IV (in forskolin) led to a clear and significant reduction in mIPSC frequency (see Fig. 5A for 1 example; P = 0.0116). To ensure maximal

activation of adenylate cyclases by forskolin, slices were preincubated in 10 \(\mu\)M forskolin for 12.6 ± 1 min (n = 5) prior to exposure to DCG-IV. As can be seen in Fig. 5E, there is a pronounced and significant increase in absolute number of mIPSCs with this procedure compared with a 2-min perfusion of forskolin (P = 0.0233).
Importantly, however, despite the significant increase in mIPSC frequency, DCG-IV still potently and significantly suppressed presynaptic GABA release after preincubation in forskolin (1 example is shown in Fig. 5B; on average 59.5 ± 11% reduction in mIPSC frequency after exposure to DCG-IV; P = 0.0089; n = 5). Figure 5, C and F (right 2 bars), shows that 0.2% DMSO, which is equivalent to the final concentration of DMSO used in the forskolin experiments, had no effect on DCG-IV’s ability to block presynaptic GABA release.

There was no significant difference in the extent of reduction of mIPSC frequency with DCG-IV obtained after preincubation or 2-min exposure to forskolin (P = 0.4107). Figure 5F therefore compares aggregate data in which the effect of DCG-IV on forskolin-mediated increase in presynaptic GABA release was tested. On average, DCG-IV reduced mIPSC frequency by 54 ± 9.9% in the presence of forskolin (left; very significant reduction with P = 0.0046). This is similar to the extent of reduction induced by DCG-IV under control conditions in the absence of forskolin (Fig. 5F, right; no significant difference between extent of reduction with DCG-IV in forskolin and DMSO only; P = 0.3918).

This suggests that forskolin-induced increases in presynaptic GABA release are subject to inhibition by activation of presynaptic group II mGluRs.

Given that activation of group II mGluRs reduces GABA release after exposure to forskolin but not to elevated K⁺, it was of interest to see whether the forskolin-induced increase in mIPSC frequency was independent of activation of presynaptic VGCCs (Kondo and Marty 1997; Yoshihara et al. 1999). Figure 6, A and C (left 2 columns), shows that this is indeed the case. Even in the presence of 200 μM Cd²⁺ and 200 μM Ni²⁺, forskolin still induced a significant increase in mIPSC frequency (88.1 ± 60.8% increase; P = 0.017) that was virtually identical to that observed in the absence of 200 μM Cd²⁺ and 200 μM Ni²⁺ (99.7 ± 33.1%; statistically significant increase compared with control with P = 0.044; Fig. 6, B and C, right 2 columns).

Taken together, these results show that the forskolin-induced increase in mIPSC frequency is independent of the activity of VGCCs.

Application of either elevated K⁺ or forskolin not only led to an increase in IPSC frequency but also an increase in average IPSC amplitude. On average, forskolin increased mIPSC amplitude by 16.7 ± 9.8% (P = 0.031; n = 13; Fig. 7A). K⁺ (15 mM) increased tIPSC amplitude by 12.4 ± 5.4% (P = 0.02; n = 27; Fig. 7B).

Figure 7, C and D, compares amplitude distributions of IPSCs recorded from Purkinje cells exposed to either forskolin (Fig. 7C) or elevated K⁺ (Fig. 7D). Amplitude histograms were normalized to total number of events recorded over a 3-min period to investigate if certain amplitudes are affected by application of forskolin/elevated K⁺ to a lesser or greater extent. Figure 7, top, shows the control distribution of amplitudes (bin width is 10 pA), whereas the middle panel shows the amplitude distribution obtained in either forskolin (Fig. 7C) or elevated K⁺ (Fig. 7D). The bottom panels show the amplitude histogram that results from subtracting the control histogram from the test histogram (i.e., top histogram subtracted from middle histogram) to uncover the forskolin/elevated K⁺-induced changes.

For both treatments, there was a decrease in number of small amplitude mIPSCs and an increase in number of larger amplitude IPSCs, showing that both forskolin and elevated K⁺ achieve an increase in average IPSC amplitude by both reducing small amplitude events and increasing large amplitude events.

The loss of small-amplitude currents and increase in bigger-amplitude currents probably reflects the fact that due to increased GABA release there is a bigger chance of two release events occurring at the same time, thereby giving rise to one big rather than two small events. Alternatively, these results could be explained by a general increase in IPSC size and a reduction in small size mIPSCs with a parallel increase in large size mIPSCs.

It was previously published that activation of presynaptic NMDA receptors on cerebellar interneurons leads to an increase in GABA release from interneurons onto Purkinje cells (Glitsch and Marty 1999). Given that both receptors use the same neurotransmitter, glutamate, it was of interest to see whether NMDA could still enhance presynaptic GABA release in the presence of DCG-IV.

Originally, experiments were designed in which first 30 μM NMDA was applied and then 5 μM DCG-IV added (analogous to elevated K⁺ experiments). However, control experiments...
showed that prolonged exposure to 30 μM NMDA alone lead to a pronounced desensitization of the NMDA response, resulting in a decrease in presynaptic GABA release by 30 ± 19% (n = 7). This reduction in presynaptic GABA release due to prolonged NMDA exposure alone was statistically not significantly different from the decrease in presynaptic GABA release observed in the prolonged presence of NMDA and additional presence of DCGIV (47 ± 21% reduction, n = 5; P = 0.547).

However, there was a concern that the desensitization of the presynaptic NMDA receptors might mask potential DCG-IV effects. Therefore experiments were repeated to verify that NMDA could still increase presynaptic GABA release after activation of presynaptic group II mGluRs. Figure 8 shows that this was the case. After a reduction in mIPSC frequency with 5 μM DCG-IV (Fig. 8A, middle), there is a clear increase in mIPSC frequency after addition of 30 μM NMDA (Fig. 8A, right). Figure 8B compares the increase in mIPSC frequency after stimulation with NMDA in the absence (right 2 bars) and presence (left 2 bars) of 5 μM DCG-IV. Application of NMDA after activation of type II mGluRs with DCG-IV resulted in a robust increase in mIPSC frequency (left ■) that was not significantly different to that observed in the absence of DCG-IV (right ■).

These results suggest that the NMDA-induced increase in GABA release, like the 15 mM K⁺-induced GABA increase, is not subject to modulation by presynaptic group II mGluRs.

**D I S C U S S I O N**

The main finding of this study is that there are two different GABA release pathways at the interneuron–Purkinje cell synapse that are differentially regulated by presynaptic group II mGluRs. The GABA release machinery of the Ca²⁺ influx-dependent pathway (activated either by terminal depolarization after application of 15 mM K⁺ or NMDA receptor activation) is not subject to inhibition by group II mGluRs, whereas the VGCC-independent pathway (spontaneous and cAMP-dependent GABA release after application of forskolin) is.

Neither spontaneous nor cAMP-dependent GABA release depend on Ca²⁺ influx through VGCCs. Indeed, in cerebellum, it has been shown that both mIPSCs and the cAMP-dependent GABA release persist in the absence of external Ca²⁺ (Kondo and Marty 1997; Llano and Gerschenfeld 1993; Llano et al. 2000). The fact that activation of group II mGluRs results in a decrease in both spontaneous and cAMP-dependent GABA release under conditions when Ca²⁺ influx and Ca²⁺ release are suppressed suggests that group II mGluRs are inhibiting a late step in the release process, probably the release machinery itself (Blackmer et al. 2001, 2005; Cartmell and Schoepp 2000; Gerachshenko et al. 2005; Offermanns 2003; Sullivan 2005).

In contrast, the increase in GABA release after stimulation with 15 mM external K⁺ or NMDA receptor activation (which...
can increase intracellular Ca\(^{2+}\) concentration by Ca\(^{2+}\) influx through the NMDA receptor itself or by depolarizing the presynaptic membrane, thereby activating VGCCs) is unaffected by stimulation of presynaptic group II mGluRs.

It is well known that AP-dependent neurotransmitter release, which recruits VGCCs, can be modulated by G-protein-coupled receptors. In theory, this inhibition of AP-dependent neurotransmitter release can be the result of modulation of ion channel function (inhibition of VGCCs or opening of K\(^{+}\) channels, which will hyperpolarize the terminal, thus making it harder for an incoming AP to trigger neurotransmitter release) and/or inhibition of the actual presynaptic release machinery (for recent reviews, see Bouron 2001; Cartmell and Schoep 2000; Miller 1998; Offermanns 2003; Sullivan 2005). Inhibitory effects of presynaptic G-protein-coupled receptors on presynaptic voltage-gated ion channels would decrease AP-evoked neurotransmitter release only without affecting the Ca\(^{2+}\) influx-independent spontaneous release (Doze et al. 1995; Gereau and Conn 1995; McQuisition and Colmers 1996; Prince and Stevens 1992).

If spontaneous and AP-induced neurotransmitter release use the same pool of synaptic vesicles and the same release sites (i.e., release machineries), as has been shown for some synapses (Murthy and Stevens 1999; Prange and Murphy 1999; Rosenmund and Stevens 1996; Ryan et al. 1997; Van der Kloot 1996 and references therein), then activation of presynaptic G-protein-coupled receptors should interfere directly and equally with the release machinery of both spontaneous and AP-evoked neurotransmitter release (as is shown in Dittmann and Regehr 1996; Nicola and Malenka 1997; Scanziani et al. 1995).

However, this was clearly not the case here. Activation of group II mGluRs affected only spontaneous GABA release. There is increasing evidence that at some synapses, spontaneous and AP-evoked neurotransmitter release may require different release machineries/vesicles for spontaneous and AP-evoked neurotransmitter release. To the author’s knowledge, this is the first study showing that the release machineries of depolarization/AP-induced and spontaneous neurotransmitter release can be differentially regulated by presynaptic G-protein-coupled receptors in the CNS.

At first glance, the results presented here may seem confusing. On the one hand, it has been shown for the interneuron–Purkinje cell synapse that both AP-induced and spontaneous GABA release are blocked after activation of group II mGluRs (Glitsch et al. 1996). However, the results of this study suggest that this block is not achieved by one common pathway, the modulation of the release machinery, but by two independent pathways: AP-induced GABA release appears to be reduced due to activation of presynaptic K\(^{+}\) channels (because VGCCs could still be activated in this study in the presence of DCG-IV), whereas spontaneous release is reduced due to effects on the release machinery. A clue to answer the question why one common pathway is not used to reduce presynaptic GABA release may lie in the NMDA experiments carried out in this study.

Activation of NMDA receptors leads to clear increases in presynaptic GABA release in cerebellar slices; this has three interesting properties: it triggers presynaptic GABA release even in the presence of TTX, suggesting that the NMDA receptors responsible for this increase are on or near the terminal; it only triggers release of GABA from inhibitory synapses, whereas glutamate release from excitatory synapses is unaffected; and it only increases the frequency of small mIPSCs, suggesting that mainly monoquantal release is affected (Glitsch and Marty 1999). On the other hand, activation of group II mGluRs, which have a very similar \(K_D\) for glutamate, suppresses presynaptic GABA release. These results are puzzling in that the same neurotransmitter can both increase and depress GABA release over a similar concentration range.

However, the present findings could resolve this paradox: glutamate will activate presynaptic group II mGluRs, thereby efficiently blocking both AP-induced and spontaneous GABA release. Spontaneous release at the interneuron–Purkinje cell synapse can be multiquantal and hence give rise to relatively large inhibitory potentials postsynaptically (Llano et al. 2000). At the same time, activation of presynaptic NMDA receptors will still be able to trigger increases in GABA release because the Ca\(^{2+}\) influx-dependent GABA release machinery is not affected by group II mGluR activation. However, NMDA receptor activation will only give rise to increases in smaller, most likely monoquantal release of GABA.

Physiologically, a likely source for glutamate activating these presynaptic receptors, are neighboring excitatory synapses. There are two excitatory inputs into Purkinje cells that use glutamate as their neurotransmitter, the parallel fiber and the climbing fiber input. These two inputs differ substantially in their ability to excite the postsynaptic Purkinje cell: while a given parallel fiber will generally make only one or a few excitatory synapse(s) with a given Purkinje cell (mainly on the more distal dendrites), a given climbing fiber will make hundreds of synapses with one Purkinje cell (proximal dendrites of the Purkinje cell). Activation of a parallel fiber can trigger a simple spike in Purkinje cells but may not always depolarize the postsynaptic Purkinje cell sufficiently to induce Purkinje cell firing. Activation of the climbing fiber, on the other hand, triggers a complex spike in Purkinje cells and usually initiates a burst of APs in the Purkinje cell. Because the climbing fiber input is the dominant glutamatergic input for Purkinje cells, it is likely that the climbing fiber–Purkinje cell synapse is the source of glutamate that activates presynaptic NMDA receptors and group II mGluRs on interneurons.

Purkinje cells display long-term depression (LTD) of the parallel fiber input when parallel fiber and climbing fiber inputs coincide. This has been shown to be the result of AMPA receptor internalization in the postsynaptic membrane of the parallel fiber–Purkinje cell synapse. As a consequence, a subsequent AP in the parallel fiber will not trigger an excitatory postsynaptic potential in the Purkinje cell because the synapse(s) in question has been silenced. This process is believed to be a powerful mechanism underlying cerebellar learning and memory formation (reviewed in Ito 2001).

With a view to the findings of this study, it is tempting to propose the following model: climbing fiber firing will result in glutamate release, which can spill over to neighboring interneuron–Purkinje cell synapses. At these synapses, binding of glutamate to presynaptic group II mGluRs will suppress both
AP-dependent and spontaneous GABA release, which can be multiquantal and hence give rise to large and somewhat variable hyperpolarizing potentials postsynaptically. At the same time, activation of presynaptic NMDA receptors will trigger increased, presumably monouquantal, GABA release, which gives rise to more uniform and smaller postsynaptic hyperpolarizations. As a net result, the postsynaptic Purkinje cell will now be left more easily excitable (but not over-excitatory due to the NMDA-induced GABA release). As a consequence of this, the firing of a presynaptic parallel fiber might now be able to trigger Purkinje cell firing. In this model, parallel fiber inputs could give rise to much bigger postsynaptic depolarizations than they normally would if they occur within a given time window after climbing fiber activity simply because the postsynaptic Purkinje cell would be in a more excitatory state.

Hence, coinciding activity of parallel fiber and climbing fiber will result in an inhibition of parallel fiber inputs via LTD, whereas a parallel fiber input that follows a climbing fiber input could give rise to much bigger postsynaptic depolarizations than they normally would if they occur within a given time window after climbing fiber activity simply because the postsynaptic Purkinje cell would be in a more excitatory state.

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REFERENCES


McQuiston AR and Colmers WF. Neuropeptide Y2 receptors inhibit the frequency of spontaneous but not miniature EPSCs in CA3 pyramidal cells of rat hippocampus. *J Neurophysiol* 76: 3159–3168, 1996.


Nikola SM and Malenka RC. Dopamine depresses excitatory and inhibitory synaptic transmission by distinct mechanisms in the nucleus accumbens. *J Neurosci* 17: 5697–5710, 1997.


