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Climbing Fiber Discharge Regulates Cerebellar Functions by Controlling the Intrinsic Characteristics of Purkinje Cell Output

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McKay BE, Engbers JD, Mehaffey WH, Gordon GR, Molineux ML, Bains JS, Turner RW. Climbing fiber discharge regulates cerebellar functions by controlling the intrinsic characteristics of Purkinje cell output. *J Neurophysiol* 97: 2590–2604, 2007. First published January 31, 2007; doi:10.1152/jn.00627.2006. The contribution of Purkinje cells to cerebellar motor coordination and learning is determined in part by the chronic and acute effects of climbing fiber (CF) afferents. Whereas the chronic effects of CF discharge, such as the depression of conjunctive parallel fiber (PF) inputs, are well established, the acute cellular functions of CF discharge remain incompletely understood. In rat cerebellar slices, we show that CF discharge presented at physiological frequencies substantially modifies the frequency and pattern of Purkinje cell spike output in vitro. Repetitive CF discharge converts a spontaneous trimodal pattern of output characteristic of Purkinje cells in vitro to a more naturalistic nonbursting pattern consisting of spike trains interrupted by short CF-evoked pauses or longer pauses associated with state transitions. All effects of CF discharge could be reproduced in the presence of synaptic blockers by using current injections to simulate complex spike depolarizations, revealing that CF-evoked changes in Purkinje cell output can occur independently of network activation. Rather postsynaptic changes are sufficient to account for the CF-evoked block of trimodal activity and include at least the activation of Ca^{2+} -dependent K^{+} channels. Furthermore by controlling the frequency of Purkinje cell spike output over three discrete firing levels, CF discharge modulates the gain of Purkinje cell responsiveness to PF inputs in vitro through postsynaptic mechanisms triggered by the complex spike depolarization. The ability for CF discharge to acutely modulate diverse aspects of Purkinje cell output provides important insights into the probable cellular factors contributing to motor disturbances following CF denervation.

INTRODUCTION

The cerebellum coordinates the execution and adaptation of motor behaviors (Ito 1984). Climbing fibers (CFs) play a central role in such behaviors by signaling via an intense complex spike excitatory postsynaptic potential (EPSP) to Purkinje cells (Simpson et al. 1996). Although the chronic effects of CF discharge on the long-term depression of conjunctive parallel fiber (PF) inputs have been well characterized (Ito 2001), the acute cellular functions of CF discharge in Purkinje cells and their underlying neuronal mechanisms remain incompletely understood. For instance, CF discharge in vivo can control the frequency and pattern of Purkinje cell spike output by punctuating tonic activity with a variable duration pause, slow the frequency of simple spike (SS) discharge, and modulate the responsiveness of Purkinje cells to

PF inputs over the short term (Barmack and Yakhnitsa 2003; Bell and Grimm 1969; Cerminara and Rawson 2004; Colin et al. 1980; Ebner and Bloedel 1981; Ebner et al. 1983; Eccles et al. 1966; Latham and Paul 1971; Loewenstein et al. 2005). The effects of CF discharge on SS activity can be further demonstrated by inactivating or removing the inferior olive, which increases SS frequency and can uncover slow oscillatory swings in Purkinje cell output (Cerminara and Rawson 2004; Colin et al. 1980; Montarolo et al. 1982; Savio and Tempia 1985). Reinstating CF input restores tonic levels of SS activity, revealing that CF input can stabilize Purkinje cell discharge in vivo. The mechanisms by which CFs reduce the frequency of SS discharge or influence PF gain has not been fully delineated. Because CF afferents have been proposed to synapse on many cell types, CF discharge may change Purkinje cell output via inhibitory interneuron networks (Barmack and Yakhnitsa 2003; Montarolo et al. 1982). Alternatively, the rich postsynaptic pattern of ion channel expression in Purkinje cells (Llinas and Sugimori 1980a,b; Raman and Bean 1997, 1999; Williams et al. 2002) suggests that CF inputs may stabilize spike output through intrinsic ionic mechanisms triggered by the postsynaptic complex spike depolarization (Hounsgaard and Midtgaard 1989; Schmolesky et al. 2002).

In the present study, we examined the effects of CF activation on Purkinje cell output in vitro, the mechanisms underlying any changes in Purkinje cell output, and its final consequences for the ability of Purkinje cells to readout PF activity. We show that CF discharge at physiological frequencies substantially modifies the frequency and pattern of Purkinje cell output in vitro. Indeed CF control over spike output is robust as the trimodal pattern of output observed in Purkinje cells in vitro (Llinas and Sugimori 1980a,b; Womack and Khodakhah 2002) is converted to a more naturalistic pattern of output in which repetitive burst discharge is no longer evident. By acutely regulating spike frequency CF discharge provides real-time modulation of the gain of Purkinje cell output in response to PF inputs. Moreover, postsynaptic mechanisms triggered by the complex spike depolarization prove sufficient to account for the effects of CF discharge on these properties as can be measured in vitro.

METHODS

Animals

Sprague-Dawley rats were purchased from Charles River Laboratories (Charles River, Quebec, Canada) and were maintained in

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standard conditions by the Animal Resources Center. Male rats \geq P18 (range: P18–P36, median = P24) were exclusively used given the adult-like physiological characteristics of Purkinje cells beyond this age (McKay and Turner 2005). All procedures were completed in accordance with the guidelines of the Canadian Council on Animal Care.

Solutions

Chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Tissue slicing and electrophysiological recordings were completed with artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 125 NaCl, 3.25 KCl, 1.5 CaCl₂, 1.5 MgCl₂, 25 NaHCO₃, and 25 D-glucose. aCSF was continuously bubbled with carbogen (95% O₂-5% CO₂) gas. For current-clamp recordings, the pipette solution consisted of (in mM) 130 K-gluconate, 0.1–10 EGTA, 10 HEPES, 7 NaCl, 0.3 MgCl₂, pH = 7.3 with KOH (0.1 mM EGTA was used for all experiments unless otherwise indicated). The characteristics of Purkinje cell spike output do not differ in a statistically significant manner between whole cell recordings completed with K-gluconate or K-methylsulfate internal solutions, which further do not differ from records of spontaneous output obtained extracellularly (unpublished observations). For voltage-clamp recordings of inhibitory postsynaptic currents (IPSCs), the pipette solution consisted of (in mM) 140 KCl, 10 EGTA, 10 HEPES, 0.1 CaCl₂, 2 MgCl₂, pH = 7.3 with KOH. To obtain voltage-clamp recordings of CF- or PF-evoked mGluR EPSCs the pipette solution contained (in mM) 140 CsCl, 5 TEA-Cl, 4 NaCl, 10 HEPES, 0.5 MgCl₂, pH = 7.3 with CsOH; 5 mM di-tris-creatine phosphate, 2 mM Tris-ATP, and 0.5 mM Na-GTP were added daily from frozen stock solutions to all pipette solutions. Picrotoxin (50 μ M), kynurenic acid (4 mM) and 6,7-dinitroquinoxalinedione (DNQX; 10 μ M; Tocris Cookson, Ellisville MO) were added to the aCSF bath solution. Tetrodotoxin (TTX; 100 nM), charybdotoxin (CbTX; 100 nM), apamin (200 nM), α -methyl-4-carboxyphenyl-glycine (MCPG; 1 mM), 7-(hydroxyimino) cyclopropa [b] chromenla-carboxylate ethyl ester (CPCCOEt; 100 μ M; Tocris), and picrotoxin (50 μ M) were applied locally with a pressure electrode containing ACSF of the following composition (in mM): 150 NaCl, 3.25 KCl, 1.5 CaCl₂, 1.5 MgCl₂, 10 HEPES, and 20 D-glucose, pH 7.4. The pressure ejection media included 0.1% BSA to minimize nonspecific toxin binding. Food coloring (1:100 dilution) was used to confirm the spatial extent of pressure-ejected drugs, and had no measurable effects alone.

Electrophysiology

Preparation of sagittal slices (300 μ m) from the cerebellar vermis and methods for current-clamp recordings in Purkinje cells have been previously described in detail (McKay et al. 2005). All recordings were completed at 35°C with no attempt to restrict recordings to any particular lobule. Purkinje cells were allowed to behave spontaneously (e.g., no applied holding current) unless otherwise indicated. For the present study, we stimulated CFs at 1 Hz or with a physiological sequence of frequencies patterned after the distribution of spontaneous CF frequencies in an awake animal (Armstrong and Rawson 1979). MatLab (MathWorks, Natick, MA) was used to create a TTL stimulus file based on this distribution that activated the stimulator through the D/A converter, and the stimulator was controlled with Clampex 8.1 or 9.0 software (Axon Instruments, Foster City, CA). During preliminary studies, we noted that Purkinje cells whose spontaneous output consisted of little if any tonic Na⁺ spike output and very high-frequency Ca-Na burst output did not respond to CF stimulation in the manners described in the following text (12 of 32 cells). Because this activity differs from past reports of age-matched recordings (McKay and Turner 2005; Womack and Khodakhah 2002), these cells were discarded and subsequent experiments focused only

on Purkinje cells the spike output characteristics of which were similar to these past reports.

Synaptic depolarizations

Direct synaptic activation of CFs and PFs was achieved using a bipolar stimulating electrode inserted into a patch pipette of <5 μ m tip diameter containing ACSF and stimuli delivered from an isolation unit (DS2 Isolation Stimulator, Digitimer Ltd, England). AMPA-mediated CF and parallel fiber responses were pharmacologically isolated with picrotoxin. CF EPSCs were evoked by a stimulating electrode in the granule cell layer ~ 40 μ m from the Purkinje cell soma and identified as all-or-nothing responses evoked directly from baseline with a peak amplitude between 4 and 6 nA (McKay et al. 2005). No evidence was obtained for smaller amplitude or graded EPSCs associated with direct activation of granule cell ascending synapses with the electrode placements used here (Sims and Hartell 2005). Parallel fiber responses were evoked with a stimulating electrode positioned in the mid molecular layer at low intensities, ensuring that synaptic responses rose directly from baseline to reduce any possibility of direct dendritic activation. The relative potential contribution of parallel fiber versus ascending granule cell axons to PF evoked responses was not determined.

Waveforms used to simulate synaptic responses were constructed by fitting the sum of two exponentials to determine the rise and decay kinetics of evoked EPSCs according to the equation

$$f(t) = A_1 e^{-t/\tau_{\text{rise}}} + A_2 e^{-t/\tau_{\text{decay}}}$$

This equation was then used to reconstruct the waveform in Matlab (as described in McKay et al. 2005). The amplitudes A_1 and A_2 were arbitrarily assigned so that functions began at 0 nA and peaked at 1 nA, thus allowing for scaling to different amplitudes as required. The CF and PF sEPSCs used were calculated previously (McKay et al. 2005). The PF sEPSC waveform was scaled to produce an EPSC amplitude of 0.2–0.4 nA corresponding to a typical peak EPSP under current clamp conditions of ~ 2.5 – 3.0 mV. The simulated synaptic current waveforms were delivered to the current clamp command input of an Axoclamp 2A amplifier using the DA output of a Digidata 1322A (Axon Instruments) and pClamp 8 software.

Measurements

Data were analyzed with Clampfit 9.0 (Axon Instruments) and Origin 7.0 (OriginLab, Northampton, MA). All stimulus artifacts were truncated prior to figure preparation. Na⁺ spikes were distinguished from Ca²⁺ spikes without pharmacology given their unique characteristics (McKay and Turner 2005). Na⁺ spike frequencies (inverse of inter-spike intervals), peak spike voltages, peak AHP (trough) voltages, and mean membrane voltages over specified intervals were calculated with Clampfit 9.0. Na⁺ spikes during up states did not appreciably alter the calculations of mean membrane potentials given the short durations of Na⁺ spikes in mature Purkinje cells (McKay and Turner 2005). Mean Na⁺ spike frequency prior to PF stimulation was calculated over the eight spikes preceding the stimulus. The mean frequency of Na⁺ spikes after PF stimulation was calculated over the first two spikes after stimulation. The accuracy of automated event detection was verified by visual inspection. Statistical analyses were completed with SPSS V.13 (SPSS, Chicago IL). Average values in the text and figures represent means \pm SE.

RESULTS

CF discharge determines the frequency and pattern of Purkinje cell output

CF discharge in vivo is known to reduce the frequency of Purkinje cell SS output and to block the emergence of a slow

oscillatory discharge in background and Purkinje cell SS activity that occurs in the absence of CF inputs (Barmack and Yakhnitsa 2003; Cerminara and Rawson 2004; Colin et al. 1980; Demer et al. 1985). Given the ages of rats selected for the current experiments, $\sim 70\%$ of Purkinje cells maintained in *in vitro* slice preparations at physiological temperatures spontaneously generate a slow trimodal oscillation consisting of a tonic Na^+ spike phase (tonic), a Ca-Na burst firing phase (burst), and a hyperpolarized quiescent phase (silent; Fig. 1A), whereas the remainder spontaneously generate a tonic Na^+ spike output without Ca-Na bursts (Llinas and Sugimori 1980a,b; McKay and Turner 2005; Womack and Khodakhah 2002). To determine the effects of CF discharge on the spontaneous output of Purkinje cells, we activated CFs with an extracellular stimulating electrode at 1 or 2 Hz, which matches the range of mean CF discharge frequencies *in vivo* (Ito 1984). As the changes in Purkinje cell output were comparable between the two frequencies, only the results of 1-Hz stimulations are described. Similarly, as the outcome of CF activation was comparable between spontaneously tonic or trimodal cells, distinctions between these groups are not further made, unless the result specifically assesses a change in the characteristics of trimodal output.

CF discharge blocks trimodal output

During repetitive CF discharge the trimodal pattern of tonic (31 ± 8.4 s), burst (10 ± 2.5 s), and silent (17 ± 4.0 s) phases

(Fig. 1A) was replaced by a bimodal pattern of tonic (31 ± 8.6 s) and silent (13 ± 4.4 s) phases (Fig. 1, B–D). Repetitive Ca-Na bursts normally present within the trimodal pattern were no longer evident ($n = 6$). By comparison, PF stimulation at 1 Hz over a large range of stimulation intensities did not elicit similar changes in Purkinje cell output (0 of 20 cells). Two different patterns of output could result from CF discharge during the tonic phase. In the first case, CF discharges resulted in only a brief pause of Na^+ spike output (Fig. 1B). Alternatively, CF discharge during the tonic phase resulted in successive transitions between periods of Na^+ spiking and quiescence (long CF-evoked pauses) wherein the onset and offset of each block of Na^+ spikes was controlled by a CF discharge (e.g., a CF-controlled bistability (Fig. 1C) (Loewenstein et al. 2005). Herein rapid CF-controlled transitions from quiescence to spiking during the tonic phase are referred to as down state to up state transitions, and the converse is referred to as up state to down state transitions.

The exact pattern of CF-controlled state transitions during the tonic phase was irregular and varied from trial to trial within the same cell, although CF activation accounted for $\sim 78\%$ of state transitions. Within the tonic phase neither mean membrane potential nor Na^+ spike frequency predicted whether CF discharge would result in sustained Na^+ spike output (Fig. 1B) or periodic transitions between activity and quiescence (Fig. 1C). There was a voltage-dependent transition in CF-evoked output to the up-state in that during the silent

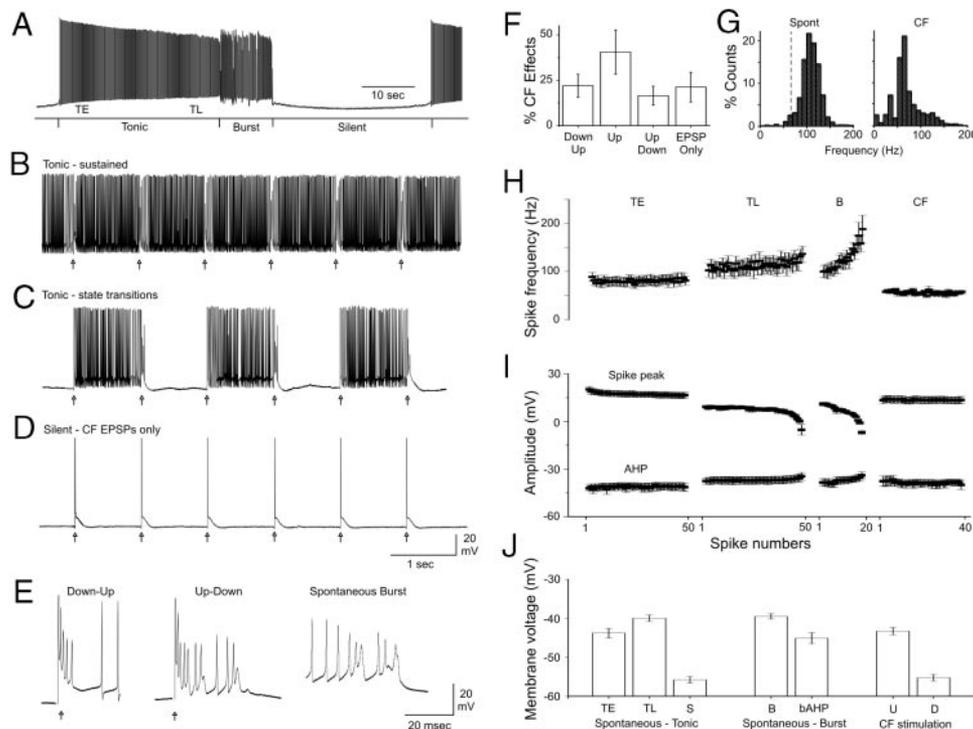


FIG. 1. Climbing fiber (CF) discharge determines the frequency and pattern of Purkinje cell output. *A*: trimodal pattern consists of a Na^+ spike phase (tonic), a Ca-Na burst phase (burst), and a quiescent phase (silent). The tonic phase is subdivided into tonic-early (TE) and tonic-late (TL). *B–D*: CF discharge at 1 Hz (\uparrow) abolishes the Burst phase of trimodal output. During the Tonic phase, CF discharge results in either sustained Na^+ spike output (*B*) or periodic transitions between up and down states (*C*). During the Silent phase, CF discharge activates complex spike excitatory postsynaptic potentials (EPSPs) only (*D*). *E*: expanded views of spike output during a CF-evoked down state to up state transition (*left*) and an up state to down state transition (*middle*). The end of a spontaneous Ca-Na burst is shown for comparison (*right*). *F*: plots of the incidence of the 4 responses to CF discharge. *G*: Na^+ spike frequency histograms for spontaneous output (*left*) and during CF discharge at 1 Hz (*right*) for a representative Purkinje cell. Dashed line, the peak frequency count of the CF histogram. *H* and *I*: average spike frequency (*H*) and peak Na^+ spike voltage (*I*, top) and AHP troughs (*I*, bottom) during trimodal activity and after initiating CF discharges. *J*: average membrane voltages during TE, TL and silent (*S*) phases of trimodal output, spontaneous Ca-Na bursts (*B*) and burst AHPs (bAHPs), and during CF-evoked up (*U*) and down (*D*) states. *A–E* and *G* are from the same Purkinje cell. Purkinje cells per data point for *F*, *H–J*: $n = 10$.

phase, CF discharges resulted only in the generation of complex spike EPSPs if the membrane potential was more negative than -56.1 ± 0.6 mV (Fig. 1D; $n = 9$). At more depolarized potentials, CF activation resulted in a full transition to the tonic phase with the patterning of spike output controlled by subsequent CF discharges as described in the preceding text. The patterning of Purkinje cell output came under CF control within ~ 20 s of 1-Hz stimulation and returned to spontaneous trimodal output over approximately the same time course when CF stimulation was stopped. Interestingly, this follows the same time course as the buildup of intracellular Ca^{2+} evoked by simulated CF discharge at 1 Hz in culture (Maeda et al. 1999) and direct CF activation in acute slices (Miyakawa et al. 1992) (explored in greater detail in the following text).

Further characterization of CF discharge during up and down states of Purkinje cell output revealed that Ca-Na bursts could still be evoked depending on firing state. From the down state, the CF evoked complex spike consisted of a principal spike overshooting 0 mV followed by several trailing low-amplitude spikelets. The complex spike was followed by a short pause, and then low-frequency Na^+ spikes that were continuously generated until the next complex spike (Fig. 1E, left). CF discharge during the up state also triggered a complex spike, but the complex spike was now followed by a brief high-frequency burst of Na^+ spikes (Fig. 1E, middle). This high-frequency burst of spikes could be followed by a sustained low-frequency spike output as occurs during a maintained up state or could be followed by a transition to a down state. A comparison of the CF-evoked high-frequency burst of spikes to single Ca-Na bursts generated during spontaneous trimodal output revealed marked similarities between the two events (Fig. 1E, right). Thus single Ca-Na bursts, although seemingly atypical of Purkinje cell output when repetitively generated during spontaneous trimodal output, appear to be an output of Purkinje cells that can be evoked singly by CF discharge.

CF discharge reduces Na^+ spike frequency and maintains stable Na^+ spike characteristics

CF discharge in vivo is known to reduce the frequency of Purkinje cell simple spike output (Barmack and Yakhnitsa 2003; Benedetti et al. 1984; Cerminara and Rawson 2004; Colin et al. 1980; Savio and Tempia 1985). We found that the frequency of Na^+ spike output in the tonic phase during CF discharges was also significantly less than that during the tonic phase of spontaneous trimodal activity (Fig. 1, G and H). To contrast the changes in successive Na^+ spikes during trimodal output with the Na^+ spikes between CF discharges, we quantified several Na^+ spike characteristics. We evaluated the first 50 (tonic-early) and last 50 (tonic-late) Na^+ spikes of the tonic phase, Na^+ spikes within single Ca-Na bursts during trimodal output, and Na^+ spikes between CF discharges (Fig. 1, H and I). Only the last 20 Na^+ spikes in each Ca-Na burst are plotted given the variability in number of spikes within Ca-Na bursts between cells. Na^+ spike frequency between CF discharges was calculated only for the low-frequency spikes; Na^+ spikes within the complex spike and the associated Ca-Na-like burst were excluded. Only the last 40 spikes between CF discharges are plotted given the variability between cells with respect to the frequency of Na^+ spike output after CF discharge.

During the tonic-early phase of trimodal activity sodium spike frequency was stable but increased dramatically at the end of the tonic-late phase and at the ends of each Ca-Na burst (Fig. 1H). By comparison, introducing repetitive CF discharges significantly decreased mean Na^+ spike frequency compared with all other phases ($P < 0.05$, $n = 10$). Paralleling the change in spike frequency during spontaneous output was a decrease in peak Na^+ spike voltage and a depolarizing shift in the AHP troughs (Fig. 1I). CF discharges abolished these changes, transforming output to maintain stable spike heights and AHP troughs throughout the duration of Na^+ spiking ($n = 10$; $P < 0.01$ compared with all other phases; Fig. 1I). Correspondingly, the membrane potentials measured during the tonic-late phase and during Ca-Na bursts were maintained at the level of tonic-early values during CF discharges (Fig. 1J).

Our results thus show that repetitive CF input abrogates repetitive Ca-Na burst discharge, thereby converting the trimodal pattern of spike output (tonic, burst, silent) found in vitro to a more stable and lower frequency of Na^+ spike discharge. Indeed, CF discharge stabilized multiple measures of Purkinje cell excitability, resulting in patterns of spike output more characteristic of Purkinje cells measured in vivo, such as a lower frequency of Na^+ spike output with CF-controlled patterning of tonic and silent phases (Bell and Grimm 1969; Colin et al. 1980; Loewenstein et al. 2005; see also Schonewille et al. 2006). The CF-evoked block of oscillatory output of Purkinje cells reported in vivo (Cerminara and Rawson 2004; Colin et al. 1980) may then correspond at least in part to a block of the trimodal activity intrinsic to Purkinje cells (Llinas and Sugimori 1980a; McKay and Turner 2005; Womack and Khodakhah 2002). The mechanisms underlying the effects of CF discharge were subsequently pursued.

Postsynaptic depolarization is sufficient to account for the effects of CF discharge

The CF-Purkinje cell synapse is glutamatergic, activating both AMPA ionotropic receptors and mGluR1 metabotropic receptors. AMPA receptor activation results in a large amplitude (several nA) EPSC or the complex spike waveform (Konnerth et al. 1990). Reports of mGluR1 receptor activation by CF input differ in terms of recording a small amplitude EPSC (Carta et al. 2006; Dzubay and Otis 2002) or either an absence or variability of this current between Purkinje cells (Reichelt and Knopfel 2002; Tempia et al. 1998; Wadiche and Jahr 2005). CFs have also been reported to excite molecular layer interneurons, which in turn inhibit Purkinje cells (Ito 1984). Thus the effects of CF activation on trimodal activity may be mediated by one or more of these mechanisms.

We began by assessing GABA- and mGluR1-mediated currents in voltage-clamp mode and then proceeded with current-clamp experiments to examine their contribution to CF-evoked changes in spike output. The high spontaneous activity of molecular layer interneurons in acute cerebellar slices (Hausser and Clark 1997) enabled us to assess GABAergic IPSCs in the absence of extracellular stimulation. To measure GABAergic IPSCs, Purkinje cells were voltage clamped at -70 mV with a KCl internal solution. Spontaneous synaptic currents occurred at 15.2 ± 3.5 Hz (Fig. 2A; $n = 4$). Application of $50 \mu\text{M}$ picrotoxin decreased the frequency of spontaneous synaptic currents to 2.2 ± 0.8 Hz ($P < 0.05$) and abolished all events

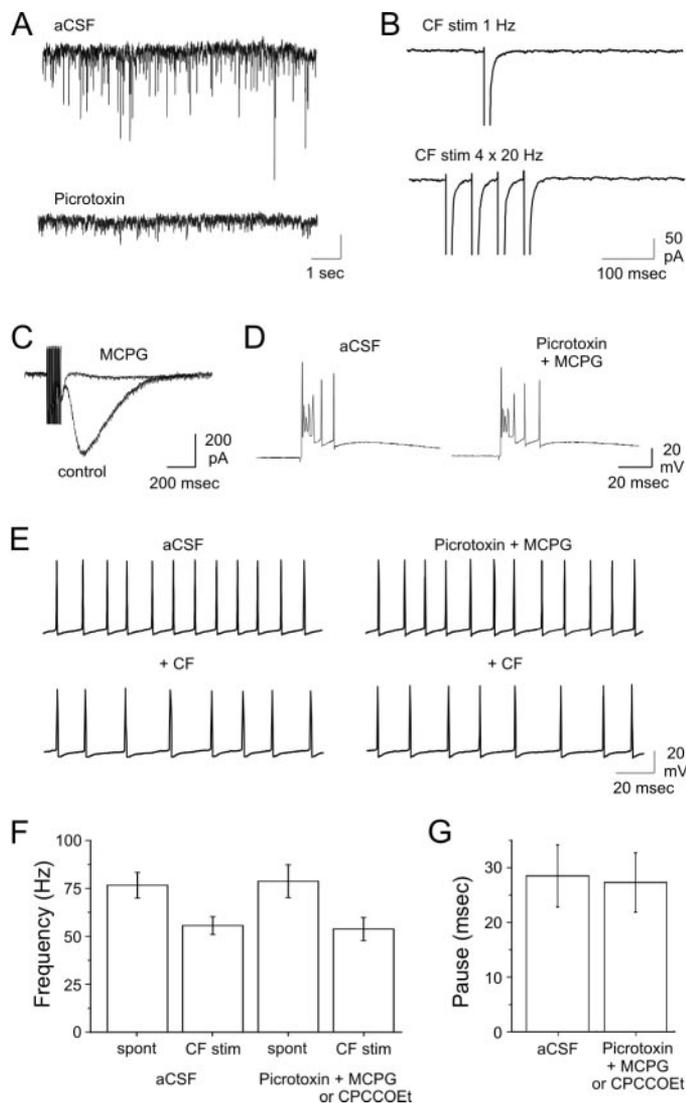


FIG. 2. Effects of CF discharge on Purkinje cell output do not require GABAergic or metabotropic glutamatergic synapses. *A*: spontaneous IPSCs in a Purkinje cell are blocked by picrotoxin. *B*: single or burst CF activation triggers a large-amplitude EPSC (truncated) but no detectable slow component consistent with mGluR activation. *C*: α -methyl-4-carboxyphenyl-glycine (MCPG) blocks mGluR-mediated slow EPSCs elicited by high-frequency PF stimulation. *D*: complex spike waveforms measured at -70 mV evoked in control artificial cerebrospinal fluid (aCSF) or following addition of picrotoxin and MCPG. *E* and *F*: CF stimulation slows the frequency of Purkinje cell spike output with or without GABA and mGluR receptor blockers. *G*: average durations of pauses in spike output after CF discharge. Recordings in *D* and *E* are from the same Purkinje cell. Purkinje cells per mean value in *F*, *G*: $n = 10$.

greater than ~ 40 pA. Thus the majority of spontaneous synaptic currents were GABAergic IPSCs, consistent with previous results (Hausser and Clark 1997).

The potential presence of mGluR-mediated CF EPSCs was assessed using either single or burst (4 stimuli at 20 Hz) CF stimulation (Dzubay and Otis 2002). Purkinje cells were voltage clamped with a CsCl-based internal solution at -70 mV in either 2 or 5 μ M DNQX to improve voltage clamp of the large AMPA receptor-mediated EPSC. In our hands, neither the single CF stimulus nor the burst protocol activated a measurable slow inward current characteristic of a mGluR response ($n = 8$ cells). This did not reflect a lack of mGluR receptors as subsequent stimulation of PFs in a subset of these recordings

elicited a robust mGluR EPSC ($n = 3$ of 3 cells; 10 stimuli at 100 Hz) (Knopfel et al. 2000; Tempia et al. 1998). We further verified the efficacy of mGluR antagonists using the PF-evoked mGluR EPSC. PF EPSCs were measured at -70 mV in Purkinje cells voltage clamped with the same CsCl-based internal solution used in the preceding text for CF-evoked responses. To improve the voltage clamp during high-frequency PF stimulation, we blocked the fast EPSC component with DNQX (10 μ M). The slow current that resulted from high-frequency PF stimulation (rise tau = 178.9 ± 39.4 ms, decay tau = 557.8 ± 84.0 ms; $n = 10$) was rapidly blocked by $\sim 95.4 \pm 1.9\%$ by MCPG ($n = 4$; Fig. 2*C*) and by $97.3 \pm 1.4\%$ by CPCCOEt ($n = 4$), drugs known to block mGluR EPSCs evoked by CF or PF stimulation (Dzubay and Otis 2002; Kim et al. 2003; Knopfel et al. 2000; Zhu et al. 2005). The reason(s) why some investigators resolve CF-evoked mGluR responses in Purkinje cells (e.g., Carta et al. 2006; Dzubay and Otis 2002), whereas others cannot (e.g., Tempia et al. 1998 and present results), is not presently known but may reflect a differential distribution of the glutamate transporter EAAT4 (Nagao et al. 1997; Wadiche and Jahr 2005).

To directly examine the contribution of GABA- and mGluR1-mediated synaptic transmission to CF-evoked changes in Purkinje cell output, CFs were activated in control aCSF and then after the addition of picrotoxin and MCPG (or picrotoxin and CPCCOEt) to the aCSF. From a hyperpolarized holding potential (-65 to -70 mV), the characteristics of the complex spike waveform were not significantly affected by any of these drug applications (Fig. 2*D*). For instance, the area of complex spikes under control conditions ($11,690.3 \pm 1,020.4$ mV \cdot ms; $n = 12$) did not change following the application of either MCPG ($n = 9$) or CPCCOEt ($n = 3$) ($11,369.9 \pm 666.4$ mV \cdot ms; $n = 12$; $P = 0.6$). Importantly, GABA and mGluR1 receptor blockade had no effect on the ability of CF stimulation to block trimodal activity or to invoke a change in the excitability of Purkinje cells (Fig. 2, *E-G*). As no differences were found between MCPG ($n = 7$) and CPCCOEt ($n = 3$) treatment, the results of the two drugs were pooled. CF activation reduced the frequency of spontaneous Na^+ spike output by 21.2 ± 4.1 Hz in control aCSF and by 25.1 ± 5.1 Hz when GABA and mGluR1 receptors were blocked (both $P < 0.01$; $n = 10$) (Fig. 2, *E* and *F*). Repetitive CF discharge blocked the Ca-Na burst component of trimodal output and revealed CF-evoked up and down state transitions even during blockade of GABA and mGluR1 receptors. Additionally, the pause in Purkinje cell spike output evoked by CF stimulation was of comparable length with or without receptor blockade (Fig. 2*G*). By comparison, all effects of CF discharge were blocked during application of the ionotropic glutamate receptor antagonist kynurenic acid (4 mM; $n = 2$).

In summary, the effects of CF discharge on Purkinje cell spike output examined here were independent of GABA- and mGluR1-mediated synaptic transmission, revealing that background and network-mediated inhibition as well as activation of metabotropic signaling cascades within the Purkinje cell are not essential to the observed effects of CF discharge. Instead the contribution of AMPA receptors to the effects of CF discharge is paramount, suggesting that the ability for CF discharge to stabilize Purkinje cell spike output relies on postsynaptic mechanisms associated with the large depolarization due to AMPA receptor activation.

Simulated CF EPSCs reproduce the effects of direct CF synaptic input

To mimic the postsynaptic depolarization elicited by extracellular CF activation, we simulated CF EPSCs (CF sEPSCs) through intracellular current injection. The waveforms of the CF sEPSCs were reconstructed from the average rise and decay kinetics of CF EPSCs measured in voltage clamp (McKay et al. 2005). A point source current injection to the soma is not a perfect substitute for the depolarization triggered by the spatially distributed CF synapse. However, we have shown previously that the complex spike EPSP generated by an intracellular CF sEPSC is very similar to the complex spike EPSP evoked by direct CF stimulation (McKay et al. 2005). We have also established that the CF sEPSC evokes long-term depression or potentiation of the PF-evoked EPSP when paired as conjunctive stimuli (1 Hz, 300 pulses; data not shown) as reported by others who used somatic current injection or postsynaptic Ca^{2+} spike discharge as the pairing stimulus (Brenowitz et al. 2006; Konnerth et al. 1992; Reynolds and Hartell 2000). The CF sEPSC delivered at the soma thus proves capable of providing the necessary changes in dendritic Ca^{2+} current to invoke PF synaptic plasticity, a result consistent with the ability for somatically generated Ca^{2+} spikes to invade extensive portions of the dendritic tree (Kano et al. 1995; Konnerth et al. 1992; Miyakawa et al. 1992; Reynolds and Hartell 2000). Therefore we used this approach to bypass the CF-Purkinje cell synapse and selectively test the effects of CF-like postsynaptic depolarizations on Purkinje cell output. Importantly, this procedure allows one to test ion channel blockers without concern for indirect effects on cell activity arising from action at presynaptic terminals (Regehr and Mintz 1994; Stuart and Sakmann 1995). To ensure no contribution of background synaptic activity to these experiments, sEPSCs

were always delivered in the presence of bath applied picrotoxin, AP5, and DNQX.

Similar to observations with extracellular CF activation, repetitive CF sEPSCs at 1 Hz converted spontaneous trimodal output (Fig. 3A) to a bimodal pattern (cf. Figs. 1 and 3). By increasing the amplitude of CF sEPSCs in 1-nA increments, we found that block of the trimodal pattern occurred over a physiological range of stimulus intensities. The range of effective CF sEPSC amplitudes was 4–6 nA (mean = 4.6 ± 0.8 nA; $n = 10$), which was similar to the size of EPSCs measured during extracellular CF activation under identical recording conditions (see McKay et al. 2005). As found for direct activation of CF input, the bimodal pattern of output during CF sEPSCs consisted of a tonic phase of sustained Na^+ spike output (Fig. 3B) or successive transitions between up and down states (Fig. 3C), and a silent phase in which CF sEPSCs evoked only complex spike-like EPSPs (Fig. 3D). The majority of state transitions ($\sim 88\%$) were again triggered by CF sEPSCs, indicating the important contribution of CF-evoked depolarizations in determining the active or quiescent state of Purkinje cells (Loewenstein et al. 2005). Transitions from the down to the up state (e.g., Fig. 3C) were characterized by a complex spike waveform followed by a short pause and then low-frequency Na^+ spikes (Fig. 3E, left). Transitions from the up state to the down state (e.g., Fig. 3C), or maintenance of the up state (e.g., Fig. 3B), were again characterized by a complex spike waveform followed by a multi-peaked event (Fig. 3E, middle) that resembled the terminal Ca^{2+} spikes discharged at the ends of spontaneous Ca-Na bursts (Fig. 3E, right). Interestingly, the CF-evoked temporary pause that follows CF activation during SS discharge in Purkinje cells was also evident when the CF sEPSC was presented during tonic discharge when not followed by a transition to the down state (Fig. 3B).

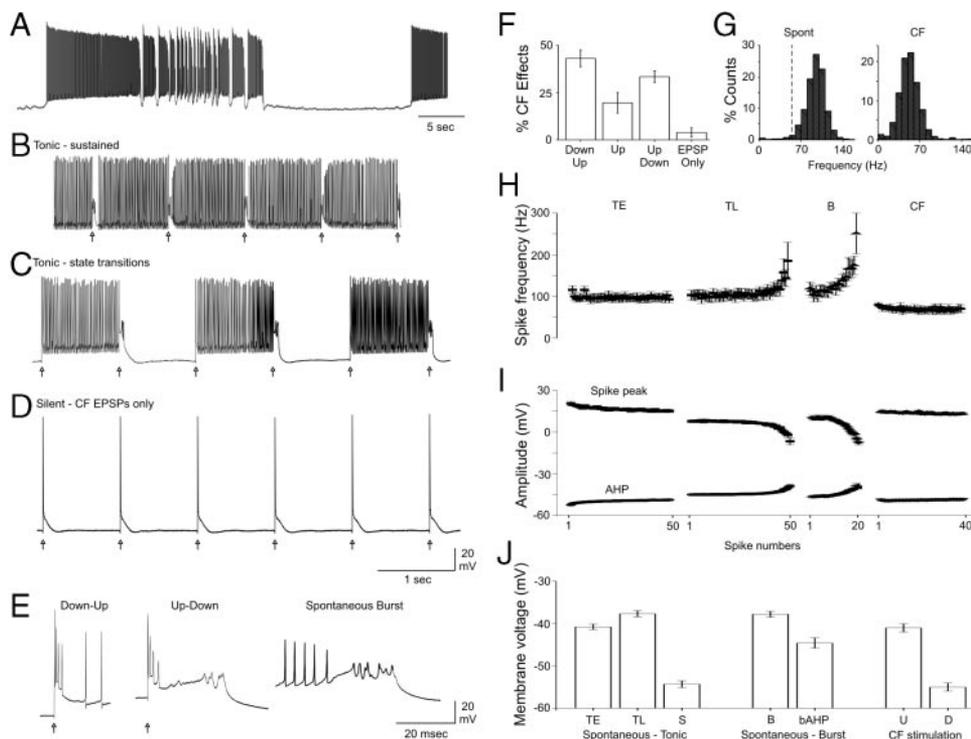


FIG. 3. Intracellular delivery of simulated CF EPSCs in the presence of synaptic blockers reproduces all the effects of CF synaptic stimulation on Purkinje cell trimodal activity and associated changes in cell output. *A*: trimodal pattern during spontaneous output. *B–D*: during the tonic phase CF sEPSCs (4 nA, 1 Hz; \uparrow) result in either sustained Na^+ spike output (*B*) or regular transitions between up and down states (*C*) and during the silent phase result in complex spike-like EPSPs only (*D*). *E*: expanded recordings during CF sEPSCs from the Purkinje cell in *A–D* illustrate spike responses during state transitions. A Ca^{2+} spike generated at the end of a spontaneous Ca-Na burst is shown for comparison. *F*: plots of the incidence of the 4 responses to CF sEPSCs. *G*: histograms of Na^+ spike frequencies for a Purkinje cell during spontaneous output (*left*) and during CF sEPSCs (*right*). Dashed line in the spontaneous plot, the peak frequency count of the CF plot. *H* and *I*: average spike frequency (*H*) and peak Na^+ spike voltage (*I*; top) and AHP troughs (*I*; bottom) during trimodal activity or CF sEPSCs at 1 Hz. *J*: average membrane voltage during trimodal activity and during CF sEPSCs. Recordings in *A–E* and *G* are from the same Purkinje cell. Purkinje cells per mean value in *F, H–J*: $n = 9$.

CF sEPSCs reduce Na⁺ spike frequency and maintain stable Na⁺ spike characteristics

CF sEPSCs prevented the acceleration of Na⁺ spike frequency seen during the tonic-late phase and during Ca-Na bursting, resulting in an overall reduction of spike frequency by $24.8 \pm 6.7\%$ ($n = 9$; Fig. 3, *G* and *H*). The percent decrease in spike frequency resulting from CF sEPSCs did not differ significantly from the decrease in spike frequency that resulted from extracellular CF activation. Moreover, the progressive decrease in peak Na⁺ spike voltages and the depolarization of AHP troughs that were apparent during spontaneous output were abrogated by intracellular CF sEPSCs (Fig. 3*I*). Additionally, CF sEPSCs blocked the slowly rising depolarization in membrane potential measured during the tonic-late phase and during Ca-Na bursts, and resulted in down state membrane potentials comparable to the silent phase (Fig. 3*J*).

Our results thus indicate that all CF-evoked changes in Purkinje cell output examined here, including the decrease in mean spike frequency, control over active versus quiescent states, and block of the trimodal pattern were entirely reproduced by CF sEPSCs, implicating a postsynaptic mechanism activated by CFs. Further, CFs are the only likely source of such large amplitude input as PF stimulation even at high intensities to simulate synchronized PF discharge did not reproduce the effects of CF discharge. Although CF activation will undoubtedly activate other network mechanisms important to Purkinje cell function, the results here firmly establish that a complex spike postsynaptic depolarization is sufficient to invoke changes associated with a stabilization of Purkinje cell output.

Na⁺ spike frequency is independent of CF discharge frequency

We have shown that repetitive CF activation at 1 Hz decreases Na⁺ spike frequency via a postsynaptic membrane depolarization. We wished to determine if the frequency of Na⁺ spike output was further sensitive to variations in the

frequency of CF discharge as would be encountered in vivo with CFs discharging in response to specific environmental stimuli or feedback inputs. Purkinje cells were thus stimulated with extracellular CF activation ($n = 2$) or with intracellular CF sEPSCs ($n = 4$) with a broad range of ISIs. The distribution of ISIs (Fig. 4*D*) was reconstructed from those reported for spontaneous CF discharge in an awake animal (Armstrong and Rawson 1979, their Fig. 4*C*). The histogram of this distribution has a peak at ~ 1.5 Hz and a range of 0.4–9.9 Hz. The ISIs within this distribution were randomly selected to generate a putatively “physiological” sequence of ISIs. As extra- and intracellular application of this protocol in vitro resulted in comparable effects on Purkinje cell output, the results were pooled.

Either direct CF activation or CF sEPSCs delivered with a physiological sequence of ISIs reduced the frequency of Na⁺ spike output, triggered transitions between up and down states, and abrogated trimodal output as found for CF activation at 1 Hz. The effect of this protocol on a single Purkinje cell is shown in Fig. 4*A*. We found that the frequency of Na⁺ spike output was not determined by the interval between CF discharges over these rates of stimulation (Fig. 4*B*). Further, we calculated the mean Na⁺ spike frequency between CF stimuli, again finding no relationship between Na⁺ spike frequency and CF discharge interval (Fig. 4*C*). Transitions between states were equally likely at all ISIs. However, if CFs were activated for several seconds at high frequency (e.g., 10 Hz), a suppression of Na⁺ spike output was noted ($n = 2$), consistent with in vivo reports (Rawson and Tilokskulchai 1981). Our results thus indicate that a physiological frequency of CF activation promotes a constant baseline frequency of Na⁺ spike output.

Ionic mechanisms underlying the block of trimodal output

The pattern of change in Na⁺ spikes during the trimodal pattern suggested a progressive spike inactivation process that leads to a repetitive Ca-Na burst mode. Similarly, during single Ca-Na bursts, the depolarization that led to a burst-terminating

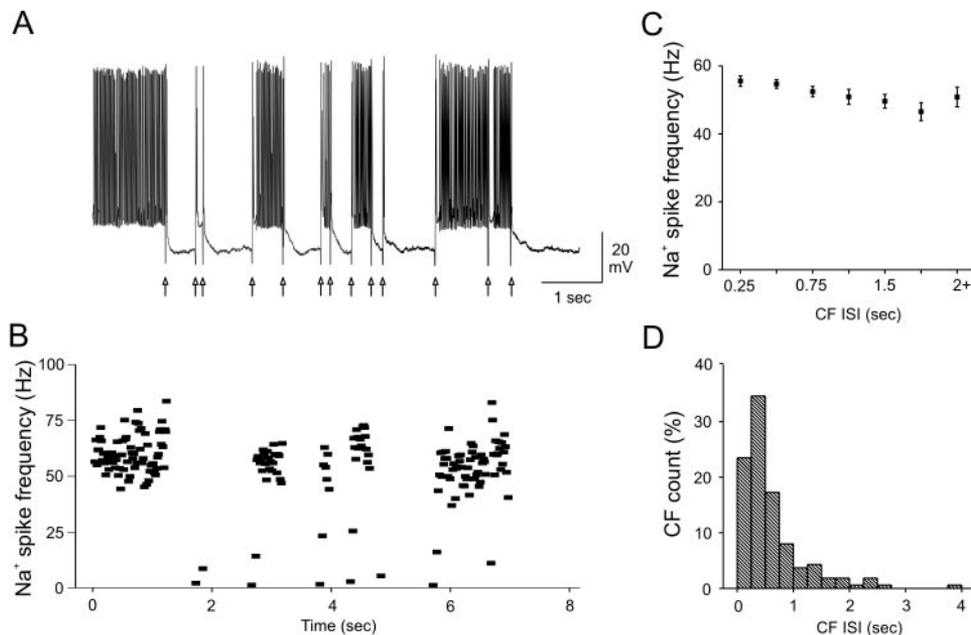


FIG. 4. Presenting a physiological range of CF discharge or CF sEPSC frequencies maintains a stable frequency of Na⁺ spike output. *A*: representative example of Purkinje cell spike output in response to CF sEPSCs at physiological frequencies (\uparrow). *B*: Purkinje cell Na⁺ spike frequency during CF discharges plotted and aligned with the recording in *A* (Na⁺ spikes in the CF-evoked high-frequency bursts are off-scale). *C*: the average frequency of Na⁺ spike output between CF discharges is not affected by the ISI of moderate CF activation (bin widths are 250 ms for CF ISIs <1 s (e.g., 0.25 s = 0–250 ms, etc) and 500 ms for CF ISIs >1 s (e.g., 1.5 s = 1,001–1,500 ms, etc)). *D*: histogram of the percent of total CF discharges at each ISI for the physiological stimulation pattern (bin width = 250 ms; reconstructed from Armstrong and Rawson 1979). Na⁺ spikes per data point for *C*: $n = 14$ –123 ($n = 6$ Purkinje cells).

Ca^{2+} spike was associated with an increase in Na^+ spike frequency, a decrease in peak Na^+ spike voltage, and a depolarization of AHP troughs. These changes likely reflect a cumulative inactivation of Na^+ channels during high-frequency output and a cumulative decrease in outward current during the AHPs. Therefore our data suggest that the duration and magnitude of the depolarization underlying both Ca-Na bursts and the Ca^{2+} spike itself may be dependent on K^+ currents triggered by Na^+ spikes. Consistent with this interpretation are data from previous reports that Ca^{2+} spikes are generated after Na^+ spike inactivation and that the magnitude of Ca^{2+} spikes and the frequency of Ca-Na burst output are regulated by voltage- or Ca^{2+} -dependent K^+ channels (McKay and Turner 2004; McKay et al. 2005; Womack and Khodakhah 2002, 2004). The following experiments were designed to test the role of Na^+ and K^+ currents in the effects of CF discharge on Purkinje cell spike output.

CF discharge effects on trimodal activity requires Na^+ spikes

As established in Figs. 1 and 2, repetitive complex spike discharge invoked by either direct CF stimulation or with a CF sEPSC resulted in Na^+ spikes with sustained high amplitudes and pronounced AHPs and blocked repetitive Ca^{2+} spike discharge. To examine the role of Na^+ spikes in setting the frequency of Ca^{2+} spike output, we quantified the frequency of Ca^{2+} spike output before and after application of 100 nM TTX. During spontaneous trimodal output, the frequency of Ca^{2+} spikes was 2.6 ± 0.5 Hz. After application of TTX, Purkinje cells spontaneously alternated between periods of repetitive Ca^{2+} spiking and periods of quiescence (McKay et al. 2005; Womack and Khodakhah 2004). During TTX application, the frequency of Ca^{2+} spikes doubled to 5.3 ± 0.7 Hz ($P < 0.01$; $n = 8$). We found, however, that CF sEPSCs delivered in the presence of TTX did not block repetitive Ca^{2+} spike output (Fig. 5A; 0 of 6 cells) in marked contrast to the effects of CF sEPSCs under control conditions (Fig. 3). In addition, in the presence of TTX, CF sEPSCs only infrequently invoked transitions from the down state to the up state, whereas transitions from the up state to the down state were not observed. These results reveal that Na^+ spikes normally regulate the frequency of Ca^{2+} spike output (presumably through the action of repolarizing K^+ currents), and that the ability for complex spike discharge to stabilize Purkinje cell output depends on intact Na^+ spike discharge.

Effects of CF discharge depend on intracellular Ca^{2+} levels

CF discharge at 1 Hz, whether elicited by synaptic activity or via simulated CF-like depolarizations, results in a prominent rise in intracellular Ca^{2+} for ~ 20 s (Maeda et al. 1999; Miyakawa et al. 1992). We thus examined the effects of intracellular Ca^{2+} buffering on the characteristics of spontaneous Purkinje cell output and the outcomes of CF discharge. We found that spontaneous output depended on the concentration of internal EGTA with significant differences between low concentrations of EGTA (0.1 or 1 mM; $n = 20$), and high concentrations of EGTA (5 or 10 mM; $n = 20$). With high concentrations of EGTA the duration of the tonic phase of the trimodal pattern was decreased by $\sim 76\%$ ($P < 0.01$) and the

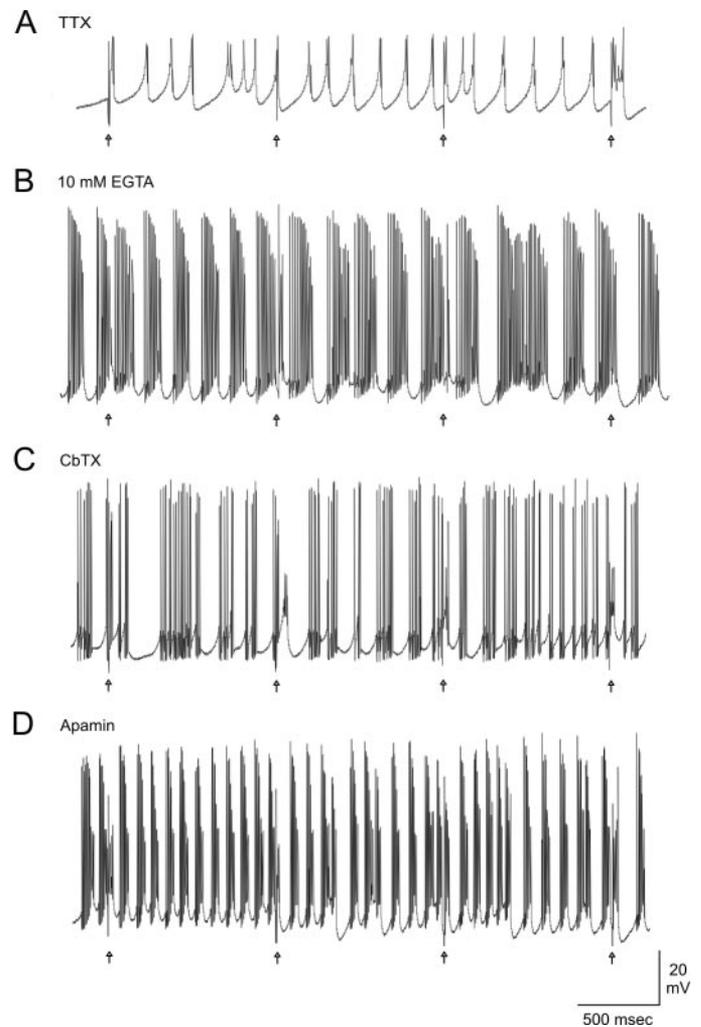


FIG. 5. Ionic mechanisms of CF discharge. A: repetitive Ca^{2+} spike output uncovered by TTX application is not arrested by CF discharge (\uparrow). B–D: intense Ca-Na burst output resulting from high internal EGTA (B) or from external CbTX (C) or apamin (D) application is unchanged by CF discharge evoked by direct activation (B) or CF sEPSC (C,D).

frequency of Ca-Na burst output was increased by $\sim 84\%$ ($P < 0.05$). Furthermore, in Purkinje cells with high concentrations of EGTA CF discharges were unable to block trimodal output, and state transitions triggered by CF discharges were rare (Fig. 5B; 7 of 7 cells). These results indicate that higher levels of intracellular Ca^{2+} (e.g., low concentrations of EGTA) help sustain Na^+ spike output with low-intensity Ca-Na burst output. Lower levels of intracellular Ca^{2+} (high concentrations of EGTA) decrease the time required to inactivate Na^+ spike output and promote higher intensity burst output. Thus the regulation of Purkinje cell spike output by CF discharges also depends on the level of intracellular Ca^{2+} .

Effects of CF discharge require Ca^{2+} -dependent K^+ channels

In many systems, K^+ currents maintain a relatively negative membrane potential by providing repolarizing currents and a background K^+ conductance. These currents contribute to setting a lower frequency of spike output and help reduce the cumulative inactivation of Na^+ channels. Our finding that

changes in the characteristics of Na^+ spike output were correlated to intracellular Ca^{2+} levels suggests a potentially important role for Ca^{2+} -dependent K^+ currents. Consistent with this idea, both large-conductance (BK) and small-conductance (SK) channels contribute to spike-activated AHPs in Purkinje cells (Edgerton and Reinhart 2003; McKay and Turner 2004; Walter et al. 2006; Womack et al. 2004). Interestingly, repetitive CF discharges or brief intracellular depolarizations result in marked rises in intracellular Ca^{2+} levels that remain elevated between 1-Hz stimuli (Maeda et al. 1999; Miyakawa et al. 1992). Such prolonged elevations in intracellular Ca^{2+} may be expected to enhance the activation of Ca^{2+} -dependent K^+ channels, thereby sustaining AHP generation and setting a lower frequency of Na^+ spike output. The activation of Ca^{2+} -dependent K^+ current by a complex spike has been shown in turtle Purkinje cells (Hounsgaard and Midtgaard 1989). Cerminara and Rawson (2004) speculated on the potential for this mechanism to account for a CF-evoked suppression of oscillatory discharge *in vivo*, but its potential role in this process has not been directly tested.

To test the prediction that Ca^{2+} -dependent K^+ channels participate in the effects of CF discharge, we applied either the BK channel blocker CbTX (100 nM) or the SK channel blocker apamin (200 nM). CF sEPSCs were used for this experiment instead of direct CF activation to bypass any possible block of presynaptic Ca^{2+} -dependent K^+ channels by the toxins that could change the characteristics of synaptic transmission (Hu et al. 2001; Robitaille et al. 1993). Changes in Ca-Na burst output in response to these or similar toxins have been described in detail previously and are not considered in detail here (Edgerton and Reinhart 2003; McKay and Turner 2004; Swensen and Bean 2003; Womack and Khodakhah 2004). Briefly, blocking BK or SK channels results in very similar (although not completely identical) changes in burst output. Application of either CbTX ($n = 5$; Fig. 5C) or apamin ($n = 5$; Fig. 5D) during spontaneous trimodal output decreased AHP amplitudes, shortened the tonic phase and increased the frequency of Ca-Na bursts (data not shown). Most importantly, we found that in the presence of these toxins, CF sEPSCs were unable to block trimodal output (10 of 10 cells). Although CF sEPSCs were able to intermittently trigger down state to up state transitions, stimulus-evoked up state to down state transitions were no longer possible. Thus the results are consistent with CF discharge arresting trimodal output by providing a Ca^{2+} influx that augments the activity of Ca^{2+} -dependent K^+ channels, which in turn prevent changes in Na^+ spike characteristics. In the presence of stable Na^+ spike characteristics, spontaneous Ca^{2+} spike discharge, and thus trimodal output, is suppressed.

In summary, we have shown that CF discharge regulates the frequency and pattern of Purkinje cell spike output and identified potential ionic mechanisms that mediate these effects. We next turned our attention to the functional consequences of these output patterns on the readout of PF inputs.

PF readout is improved during low-frequency spike output maintained by CF discharges

Numerous studies have documented the ability for CF input to provide short-term modulations of Purkinje cell SS discharge and the response to mossy fiber-PF input. The net effect

of CF activation *in vivo* proves to be complex with most reports of an immediate (<200 ms) or slow inverse modulation of SS activity (Barmack and Yakhnitsa 2003; Benedetti et al. 1984; Cerminara and Rawson 2004; Colin et al. 1980) and an increase in magnitude (gain) of the Purkinje cell SS response to mossy fiber afferent inputs (Ebner and Bloedel 1981). The source of these changes in SS activity has not been fully determined but is often attributed to the depolarizing influence of the complex spike ("inactivation response") or inhibitory interneuron activation (Barmack and Yakhnitsa 2003; Bell and Grimm 1969; Hounsgaard and Midtgaard 1989; Montarolo et al. 1982; Simpson et al. 1996). We were interested if the block of trimodal activity and shift in postsynaptic membrane excitability produced by the complex spike depolarization might affect the ability for PF inputs to activate Purkinje cells. We thus compared the relative efficacy of PF depolarizations on Purkinje cell discharge (gain) during trimodal activity and after complex spike depolarizations had instilled a lower rate of tonic Na^+ spike output. We further assessed the extent to which any effects of CF stimuli could be accounted for by changes in postsynaptic excitability.

PF inputs are most effective during low frequencies of Na^+ spike output

We first established the gain of directly evoked PF input on Purkinje cell output during spontaneous trimodal discharge (Fig. 6, A–C). Holding current was adjusted to maintain spontaneous activity of Purkinje cells at low frequency (59.4 ± 5.9 Hz; $n = 14$) or high-frequency (130.2 ± 7.8 Hz; $n = 10$) or in a burst output mode (frequency of all intra-burst Na^+ spikes: 162.6 ± 21.0 Hz; $n = 11$), and PFs were activated with extracellular stimulation. The average size of a single EPSP at -60 mV was 2.6 ± 0.2 mV ($n = 24$), an amplitude below threshold for Na^+ spike generation and activation of the long mGluR-mediated depolarization that can be triggered by more intense PF stimulation. PFs were activated at 1 Hz to avoid EPSP summation.

During the tonic phase of trimodal activity, we could resolve PF stimulation as a marked decrease in ISI that persisted below baseline intervals over the next one to five spikes. The shortest ISI of PF-evoked Na^+ spike output occurred either between the first and second spikes or the second and third spikes. For this reason, ISIs preceding and following PF stimulation were binned into pairs. During low-frequency spontaneous activity, a single PF stimulus increased spike output from 59.1 ± 5.5 to 110.8 ± 14.5 Hz ($P < 0.01$; $n = 14$; Fig. 6A). At a higher frequency of spike output, the same sized PF stimuli increased Purkinje cell output from 133.7 ± 8.0 to 166.3 ± 9.5 Hz ($P < 0.01$; $n = 10$; Fig. 6B). This net increase was significantly less than that evoked by PF inputs during low frequency discharge ($P < 0.05$; Fig. 6G). Because the frequency of Na^+ spikes increased throughout each Ca-Na burst (Fig. 1H), it was impossible to discern within a single burst the exact effect of a PF stimulus on spike frequency. To overcome this, we compared the instantaneous frequency of each spike in a control burst (no PF stimulus) to its corresponding spike in a subsequent burst that received a PF stimulus. We found that PF stimulation during Ca-Na bursts increased spike frequency by 23.3 ± 5.0 Hz ($P < 0.01$; $n = 11$; Fig. 6, C and G). This net difference was added to the mean frequency of all intraburst

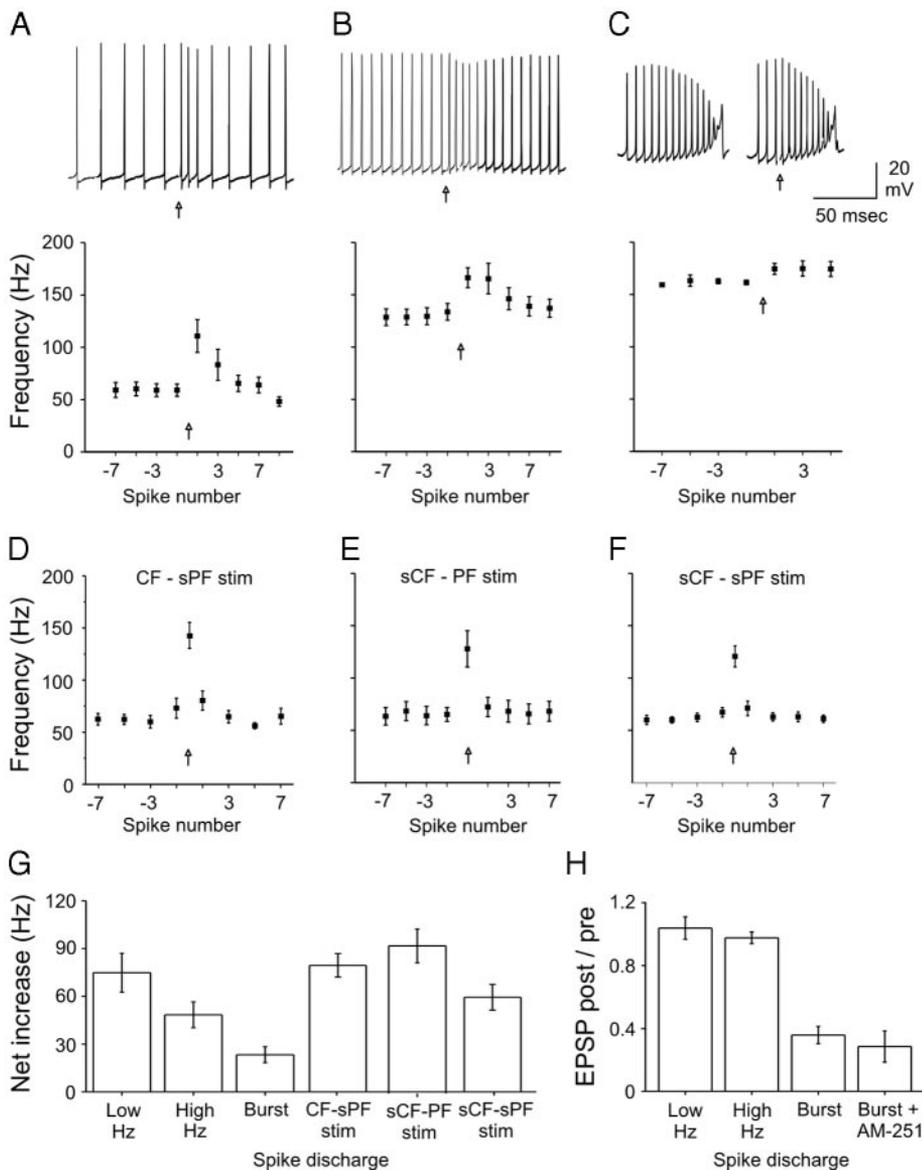


FIG. 6. Readout of PF inputs is enhanced at low spike frequencies. *A–C*: representative recordings and plots of spike output frequency when PF stimuli (\uparrow) were delivered during low-frequency (~ 60 Hz; *A*), high-frequency (~ 130 Hz; *B*), and burst (~ 160 Hz; *C*) output. In this and subsequent graphs, spikes preceding and following PF stimuli are assigned numbers <0 and >0 , respectively. PF stimulation increases spike output more at low (*A*) vs. high (*B*) spike frequencies. *C*: control Ca-Na burst (*left*) vs. a Ca-Na burst receiving PF stimulation (*right*). *D–F*: effects of PF stimuli delivered between 1-Hz CF stimuli on spike frequency after CF stabilization of Na^+ spike output. When necessary, slight bias current was applied to maintain a comparable baseline firing rate of ~ 60 Hz to facilitate comparisons. Paired stimuli consisted of direct CF synaptic input paired with PF sEPSCs (*D*), CF sEPSCs paired with direct PF synaptic input (*E*), and finally CF sEPSCs with PF sEPSCs (*F*). *G*: plots of the net increase in Na^+ spike frequency evoked by PF stimuli during spontaneous trimodal activity or with different CF-PF stimulus pairs. *H*: ratio of EPSP amplitude at -60 mV preceding and following 10-s trains of spike output to test for DSE. Purkinje cells per mean value for *A*: $n = 14$; *B*: $n = 10$; *C*: $n = 11$; *D*: $n = 4$; *E*: $n = 9$; *F*: $n = 6$; *G*: $n = 4$ – 14 ; *H*: $n = 4$ – 10 .

spikes (162.6 Hz) to more readily compare the effects of PF stimulation during Ca-Na bursts to the effects of PF stimulation during low- and high-frequency tonic output. This comparison identified a clear loss of efficacy for PF stimuli with increasing rates of Purkinje cell spike output (Fig. 6, *A–C* and *G*).

PF inputs are efficacious during low-frequency Na^+ spikes maintained by CF discharges

Our results would predict that PF readout might also be affected during the tonic low-frequency discharge of Na^+ spikes maintained during CF stimulation or the high-frequency discharge in the complex spike. We thus used direct extracellular stimulation to evoke CF EPSPs at 1 Hz to stabilize Purkinje cell output and tested the effects of a simulated PF EPSP (PF sEPSC; see METHODS; Fig. 6*D*). All experiments were done in the presence of picrotoxin. PF sEPSCs were activated ~ 500 ms following the CF sEPSC. Although this is not likely a physiological frequency of PF activation, a CF-PF pairing interval of ~ 500 ms avoids CF-triggered PF LTD (Ito 2001)

and thus allows unambiguous determination of the effects of PF stimulation during a Purkinje cell spike train. These experiments revealed that once repetitive 1-Hz CF stimulation had blocked the trimodal pattern and stabilized spike discharge, activation of a PF synaptic depolarization increased spike output from 63.4 ± 5.3 to 142.7 ± 12.5 Hz ($P < 0.01$; $n = 4$), a value strikingly similar to that observed during low frequency firing in trimodal activity (Fig. 6, *A* and *D*). To test the ability for the complex spike depolarization per se to affect PF gain, we next blocked trimodal activity with 1-Hz CF sEPSCs and tested with synaptically evoked PF EPSPs (Fig. 6*E*). In the presence of the postsynaptic complex spike response and the resulting low-frequency Na^+ spike discharge, PF inputs increased spike output from 64.6 ± 7.1 to 153.5 ± 15.3 Hz ($P < 0.01$; $n = 9$; Fig. 6, *E* and *G*). Notably, this net increase did not differ from the PF-evoked increase in frequency during spontaneous low-frequency output or in the presence of direct CF stimulation (Fig. 6*G*). In an additional three Purkinje cells we activated PFs at variable latencies relative to the CF sEPSC. PF activation during the complex spike or during the burst follow-

ing the complex spike had little or no effect on Na^+ spike output. Further, when CF sEPSCs switched Purkinje cells to a down state, the small PF inputs were insufficient to reach spike threshold. Finally, to more fully determine the role for postsynaptic mechanisms in establishing PF gain, we used CF sEPSCs to stabilize Purkinje cell output and PF sEPSPs through DC injection to measure gain. Presenting a PF sEPSC during low-frequency Na^+ spike discharge induced by repetitive 1-Hz CF sEPSCs again increased spike output from a baseline level of 62.7 ± 3.8 to 121.9 ± 10.2 Hz ($P < 0.01$; $n = 6$; Fig. 6F). The gain measured in response to simulated or direct PF stimuli in relation to CF stimuli (direct or simulated) were not significantly different from that during low-frequency spontaneous activity or from each other (Fig. 6G; 1-way ANOVA, Tukeys HSD).

These results reveal a prominent contribution of postsynaptic membrane excitability in determining PF readout that can be invoked by the complex spike depolarization with a significant decrease in efficacy for PF stimuli with increasing rates of baseline firing. Although a simple saturation of the frequency-current relationship would be a parsimonious explanation for this phenomena, Purkinje cells have a linear frequency-current relationship within this range of firing frequencies (McKay and Turner 2005). This instead suggests that the effect on PF readout with an increase in baseline firing may be due to an effective decrease in PF efficacy rather than to a saturation of the spike generating mechanism. Such a decreased efficacy of PF inputs during high-frequency tonic and burst output might be related to a presynaptic change in transmitter release due to a cannabinoid-mediated depolarization-induced suppression of excitation (DSE).

To test for DSE we compared the amplitude of PF EPSPs at -60 mV (e.g., in the absence of spike output) within 0.5 s of the onset or offset of 10 s of membrane depolarization that resulted in either low-frequency tonic, high-frequency tonic, or burst output. Compared with baseline values, PF EPSP amplitude did not change following low ($n = 8$)- or high ($n = 7$)-frequency output but was reduced following bursting ($P < 0.01$; Fig. 6H). However, this decrease in PF EPSP amplitude was not blocked by the CB1 receptor antagonist AM-251 ($5 \mu\text{M}$; $P = 0.8$; $n = 4$; Fig. 6H), ruling out DSE. In this regard, it was recently shown that 15 s (but not 5 s) of current-evoked burst activity can release endocannabinoids and produce DSE of PF inputs (Brenowitz et al. 2006). By comparison, another report concluded that only Ca^{2+} spikes triggered by intense PF activation, and not Ca^{2+} spikes generated by direct depolarization through the patch electrode, are capable of eliciting PF DSE (Rancz and Hausser 2006). Our results indicate that PF EPSPs can be decreased in a DSE-independent fashion for the 10-s time frame of burst discharge tested here.

An alternative explanation for the decreased efficacy during high-frequency spike discharge is a postsynaptic change in membrane input resistance due to the opening of voltage-gated ion channels. Such a result would be consistent with the minor effect on spike output by the PF depolarization during Ca-Na bursts or the complex spike. However, as it not possible to obtain a valid measurement of input resistance during spike discharge, the potential for this factor to influence PF gain will require further investigation.

DISCUSSION

CF activation *in vivo* is known to influence the rate of SS activity in Purkinje cells, whereas its removal can increase the rate of SS discharge and eventually uncover a slow oscillatory discharge (Barmack and Yakhnitsa 2003; Cerminara and Rawson 2004; Colin et al. 1980; Montarolo et al. 1982; Savio and Tempia 1985). A second important function of CF discharge is to change the responsiveness or gain of Purkinje cell responses to mossy fiber activation by peripheral inputs (Ebner and Bloedel 1981; Ebner et al. 1983; Latham and Paul 1971). The current work reveals that CF activation *in vitro* blocks a spontaneously active trimodal pattern of discharge in Purkinje cells to restore a more naturalistic pattern of output. Moreover, the complex spike postsynaptic depolarization *per se* is sufficient to invoke these changes and to provide the important functional outcome of improving the readout of PF inputs.

Purkinje cell discharge in vitro

A long-standing issue in the field of Purkinje cell physiology is reconciling the different activity patterns observed between *in vitro* and *in vivo* preparations. Indeed, spontaneous Purkinje cell output differs between each of the *in vitro* preparations (e.g., acute dissociation vs. organotypic culture vs. acute slice) and from spontaneous Purkinje cell output *in vivo* (Armstrong and Rawson 1979; Bell and Grimm 1969; Cavalier and Bossu 2003; Latham and Paul 1971; Llinas and Sugimori 1980a; Loewenstein et al. 2005; Raman and Bean 1999; Schonewille et al. 2006). Recent evidence indicates that some of these differences can be explained by postnatal Purkinje cell development (McKay and Turner 2005; Womack and Khodakhah 2002). However, developmental differences could not account for the persistent trimodal output in mature Purkinje cells *in vitro*. The results of the current study show that the lack of spontaneous CF input in *in vitro* slice preparations can reveal the intrinsic trimodal activity of Purkinje cells, and that reintroducing this input blocks the trimodal output to restore a low frequency of Na^+ spike discharge. Interestingly, the postnatal onset of trimodal discharge coincides with the physiological maturation of the CF-Purkinje cell synapse, revealing that CF suppression of the trimodal pattern likely occurs throughout postnatal development (Hashimoto and Kano 2005; McKay and Turner 2005).

These results are similar to those found *in vivo*, where removal of CF input uncovers an increase in SS discharge rate or even a slow oscillatory pattern of background and Purkinje cell SS discharge that returns to tonic output when CF activity is reinstated (Cerminara and Rawson 2004; Colin et al. 1980; Montarolo et al. 1982; Simpson et al. 1996). The source of these oscillations and the mechanisms underlying the stabilizing influence of CFs are difficult to address *in vivo* given that CF discharge can change Purkinje cell output directly through excitatory synaptic depolarization or indirectly through activation of inhibitory interneurons and the cortical network. The present study reveals that the ability for CF input to restore tonic discharge *in vivo* is due at least in part to a block of the mechanisms that give rise to an intrinsic trimodal pattern of Purkinje cell output.

A key tool in investigating the mechanisms underlying these effects was the use of a simulated CF EPSC to evoke a complex spike-like depolarization from the level of the soma. It is important to acknowledge that we do not expect this

response to fully replicate the actions of a complex spike evoked by synaptic input (Reynolds and Hartell 2000). However, we note that because the CF sEPSC was constructed from the net currents detected at the soma in response to direct CF stimulation, it can be expected to be accurate for somatic membrane (as supported by the high similarity of complex spikes evoked by either stimulus) (McKay et al. 2005). Previous studies have also compared the spatial extent of dendritic activity and the degree of increase in internal Ca^{2+} concentration evoked by CF activation or Ca^{2+} spikes triggered by direct somatic depolarization (Brenowitz et al. 2006; Miyakawa et al. 1992; Reynolds and Hartell 2000). These studies indicate that Ca^{2+} spikes triggered by a somatic depolarization exhibit dendritic backpropagation and an increase in internal Ca^{2+} concentration that are comparable to or even greater than that from a CF-evoked complex spike. The complex spike evoked by the CF sEPSC can then be expected to conduct to dendritic regions and invade at least as much membrane as a directly evoked complex spike. Moreover, the entirely comparable results obtained using direct CF activation or the CF sEPSC on blocking trimodal activity (Figs. 1 and 3), numerous aspects of Na^+ spike discharge (Figs. 1, 3, and 4), bistable toggling of spike output (Figs. 1, 3, and 4), and shifts in PF gain (Fig. 6) indicate that the CF sEPSC is sufficient to reproduce and test all the effects of CF activation examined here. We also used PF sEPSCs to test for postsynaptic contributions to the gain of PF input. Given the relatively small amplitude of these subthreshold responses and the extensive load imposed by Purkinje cell dendrites, we expect simulated PF EPSPs to most accurately test excitability in the soma/axon hillock region. Nevertheless, as apparent here, this approach was sufficient to reveal postsynaptic contributions to shifts in PF gain.

Purkinje cells have also been reported to exhibit a bistable membrane in vitro, where brief depolarizations can trigger longer lasting plateau depolarizations (Jaeger and Bower 1994; Llinas and Sugimori 1980a; Raman and Bean 1999; Williams et al. 2002). More recently this property has been shown to allow CF inputs to produce a toggling between up and down states both in vitro and in vivo (Loewenstein et al. 2005, 2006; Williams et al. 2002). The prevalence of this activity in vivo is controversial, with the pattern of tonic and silent phases of Purkinje cell output affected by CF input under at least some conditions of anesthesia (Bell and Grimm 1969; Loewenstein et al. 2006; Schonewille et al. 2006) but potentially also unanesthetized animals (Armstrong and Rawson 1979). The more consistent report has been one of sporadic or tonic SS activity in vivo that responds in a reproducible fashion to mossy fiber input (Barmack and Yakhnitsa 2003; Ebner and Bloedel 1981; Ebner et al. 1983; Simpson et al. 1996). The current work does not further establish the potential for CF-evoked up or down state transitions to occur in vivo but does corroborate previous reports of bistable activity in Purkinje cells in vitro that could enable this process in the intact system. As found by others using postsynaptic square wave depolarizations, we show that CF sEPSCs in the presence of synaptic blockers are sufficient to demonstrate the key role of postsynaptic membrane in controlling this activity. The alternation between tonic and silent phases in vivo does not occur with the strict regularity seen in vitro. We suspect that additional network elements in vivo may help account for this discrepancy, although other properties of membrane bistability will

also be important here (F. R. Fernandez and R. W. Turner, unpublished observations). It is also unclear whether the tonic-silent alternation in vivo includes the underlying oscillation of membrane voltage seen in vitro as this activity would be difficult to detect using extracellular recordings in vivo. Nevertheless, our results reveal that all components of trimodal output (Na^+ spike output, quiescence, and single Ca-Na bursts) and bistable toggling into up or down states are under the control of CF discharge. Together, the available evidence emphasizes that CF input serves as an important stabilizing influence on Purkinje cell output and that this can be attributed in large part to events associated with the postsynaptic complex spike depolarization.

Mechanisms underlying the stabilizing effects of CF discharge on Purkinje cells

Synaptic activation of CF afferents, as well as brief intracellular CF-like depolarizations, result in prolonged increases in Ca^{2+} concentrations (Maeda et al. 1999; Miyakawa et al. 1992). The Ca^{2+} flowing into Purkinje cells triggers a net outward current through Ca^{2+} -dependent K^+ currents, which has been shown to regulate spike and burst output (Edgerton and Reinhart 2003; Hounsgaard and Midtgaard 1989; McKay and Turner 2004; Raman and Bean 1999; Womack and Khodakhah 2002, 2004; Womack et al. 2004). In the present study, we show that the effects of CF discharge on the stabilization of spike output are also dependent on the complex spike depolarization, internal Ca^{2+} levels, and Ca^{2+} -activated outward currents. Such a mechanism was recently discussed but not tested by Cerminara and Rawson (2004). In this regard, it has been shown that a complex spike in vitro triggers a long-lasting and Co^{2+} -sensitive hyperpolarization that can build with repetitive CF activation (Hounsgaard and Midtgaard 1989). Afterhyperpolarizations reflect Ca^{2+} entry through P/Q-type Ca^{2+} channels (Womack et al. 2004), and both SK and BK K^+ channels contribute to the spike afterhyperpolarization (McKay and Turner 2004). In fact, it was recently shown that activation of SK K^+ channels promotes an increase in the precision of Purkinje cell spike output by reducing the variability in interspike intervals (Walter et al. 2006). In this regard, increases in the internal concentration of Ca^{2+} have been shown during Na^+ spike discharge at the somatic level (Lev-Ram et al. 1992). Our results further indicate that activation of Ca^{2+} -dependent outward currents important to stabilizing Purkinje cell discharge comes about through Ca^{2+} entry associated with both the complex spike and Na^+ spike discharge. This is in keeping with previous suggestions that Ca^{2+} influx during spike discharge in Purkinje cells may primarily serve to activate Ca^{2+} -dependent K^+ current to establish the rate of tonic spike discharge (Raman and Bean 1999; Williams et al. 2002).

Current data then suggest that CF activation raises intracellular Ca^{2+} through the combined action of Ca^{2+} and Na^+ spike activity to promote increased BK and SK channel activation. Na^+ spikes in turn help sustain sufficient Ca^{2+} influx to promote the activation of Ca^{2+} -dependent K^+ currents, thereby preventing the slow membrane depolarizations that lead to Na^+ spike inactivation and repetitive Ca-Na bursts. Thus a balance of inward and outward currents are implicated in the effects of CF discharge on the block of trimodal activity and stabilization of Na^+ spike output.

Cellular functions of CF discharge

From the earliest recordings of Purkinje cells, complex spikes were noted for their ability to alter the pattern and frequency of SS discharge (Armstrong and Rawson 1979; Bell and Grimm 1969; Latham and Paul 1971; Llinas and Sugimori 1980a). Extensive work has established an inverse relationship between CF and SS discharge in the form of an immediate pause in SS discharge (originally referred to as the "inactivation" response) ≤ 200 ms after a complex spike, or over even longer intervals in response to sensory input (Barmack and Yakhnitsa 2003; Simpson et al. 1996). Others addressed the ability for complex spikes to augment the responsiveness or gain of Purkinje cells in vivo to the inhibitory or excitatory effects of mossy fiber-PF input (Ebner and Bloedel 1981; Ebner et al. 1983). Beyond these reversible effects of CF discharge, extensive plasticity of synaptic transmission has been shown in the case of conjunctive CF and PF activation (Ito 2001). Indeed it has become apparent that the interactions between CFs and PFs are complex and can vary with the intensity, frequency, or timing of PF inputs with respect to CF input.

By exciting CF afferents while inhibitory networks were blocked and by using CF sEPSCs to directly excite the Purkinje cell membrane, the current study has shown that many of the short-term interactions between CF and PFs can be accounted for in terms of the postsynaptic effects of the complex spike depolarization. Thus pairing PF EPSPs with the Ca-Na spike burst of trimodal activity or the complex spike response revealed little change in PF-evoked Na^+ spike output. Although not directly testable, it can be expected that input resistance will be substantially lower during these intense spike responses, accounting for some portion of the originally described "inactivation response." We also found that a CF sEPSC evoked a temporary pause of Na^+ spike discharge (cf. Figs. 1B and 3B) similar to that of a directly evoked CF EPSP (Fig. 1). These results would indicate a postsynaptic contribution to the CF-evoked pause; the most likely effector being a Ca^{2+} -dependent K^+ current (Hounsgaard and Midtgaard 1989). The potential relationship between the CF sEPSC-evoked pause and the stabilization of Purkinje cell discharge during repetitive complex spike depolarizations is currently unknown.

Understanding the full impact of how CFs can affect PF gain is beyond the scope of the present study. A recent study established a linear relationship between the strength of PF synaptic input and Purkinje cell discharge (Walter and Khodakhah 2006). A direct comparison between these data and the current results is difficult as our recordings were performed with a single strength PF input in relation to the presence or absence of CF input and the resulting baseline frequency of Na^+ spike discharge. We have established a relationship between the rate of Na^+ spike discharge and the effective gain of PF evoked EPSPs that was similar for both spontaneous rates of Na^+ spike output and those maintained during repetitive CF activation. Our results thus suggest that CF discharge regulates the gain of the responsiveness of Purkinje cells to PF inputs over three levels: a high gain period during low-frequency spike output, a low gain period during post complex spike burst output, and a zero gain period during down states. The findings then complement previous reports that one important CF func-

tion is to control the gain of the response of Purkinje cells to mossy fiber-PF inputs in vivo (Ebner and Bloedel 1981; Ebner et al. 1983). Moreover, the CF sEPSC reproduced the correlation between the rate of tonic spike discharge and the effective gain of either evoked PF EPSPs or the PF sEPSC. These simulated EPSC tests are particularly valuable in revealing that gain can also be affected by factors independent of synaptic or metabotropic receptor effects (Walter and Khodakhah 2006). This is not to say that other synaptic or network mechanisms associated with CF or conjoint CF-PF synaptic activation will not add additional factors to consider when assessing PF gain. However, the preceding work does indicate that the complex spike depolarization makes a significant contribution to the change in PF gain observed with CF activation. This would signify a new function for CF inputs in the acute regulation of PF readout with implications for ongoing cerebellar functions. Interestingly, the frequencies of CF discharge are correlated to specific behaviors. For instance, high-frequency CF discharge is associated with learning of conditioned eye blink responses, whereas lower frequencies are associated with extinction of this behavior (Medina et al. 2002).

Role of CFs in motor learning and cerebellar ataxias

In Marr-Albus models of cerebellar function, CFs are thought to teach Purkinje cells optimal PF input patterns (Albus 1971; Marr 1969). Following sufficient CF instruction, Purkinje cells should generate correct spike responses to future PF inputs in the absence of further CF inputs. Experimental tests of these models have relied on training animals with subsequent blockade of CF discharge prior to testing for learning. Existing data, however, suggest that the loss of CF input will result in fundamental changes in spontaneous Purkinje cell behavior and readout of PF inputs. As such, experimental examination of these models may require alternative strategies. Likewise, we expect these cellular changes to contribute significantly to the observed deficits in motor coordination after CF denervation. In fact, cerebellar ataxias and impairments in some types of motor learning in humans are associated with marked atrophy of the inferior olive (Llinas et al. 1975; Manto 2005; Martin et al. 1996). Our findings may point to the acute cerebellar slice preparation as a useful model to study the cellular, synaptic and network consequences of inferior olive lesions and CF denervation, and by extension, interventions directed at restoring normal activity.

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