Cellular/Molecular

Optogenetic Visualization of Presynaptic Tonic Inhibition of Cerebellar Parallel Fibers

Ken Berglund, Lei Wen, Robert L. Dunbar, Guoping Feng, and George J. Augustine

Department of Neurobiology, Duke University Medical Center, Durham, North Carolina 27710, Departments of Neurosurgery and Anesthesiology, Emory University School of Medicine, Atlanta, Georgia 30322, Center for Functional Connectomics, Korea Institute of Science and Technology, Seoul 136-791, Republic of Korea, Lee Kong Chian School of Medicine, Nanyang Technological University, Research Techno Plaza, Singapore 637553 Singapore, Institute of Molecular and Cell Biology, Singapore 138673, Singapore, and MBL, Woods Hole, Massachusetts 02543

Tonic inhibition was imaged in cerebellar granule cells of transgenic mice expressing the optogenetic chloride indicator, Clomeleon. Blockade of GABA_A receptors substantially reduced chloride concentration in granule cells due to block of tonic inhibition. This indicates that tonic inhibition is a significant contributor to the resting chloride concentration of these cells. Tonic inhibition was observed not only in granule cell bodies, but also in their axons, the parallel fibers (PFs). This presynaptic tonic inhibition could be observed in slices both at room and physiological temperatures, as well as in vivo, and has many of the same properties as tonic inhibition measured in granule cell bodies. GABA application revealed that PFs possess at least two types of GABA_A receptor: one high-affinity receptor that is activated by ambient GABA and causes a chloride influx that mediates tonic inhibition, and a second with a low affinity for GABA that causes a chloride efflux that excites PFs. Presynaptic tonic inhibition regulates glutamate release from PFs because GABA_A receptor blockade enhanced both the frequency of spontaneous EPSCs and the amplitude of evoked EPSCs at the PF-Purkinje cell synapse. We conclude that tonic inhibition of PFs could play an important role in regulating information flow through cerebellar synaptic circuits. Such cross talk between phasic and tonic signaling could be a general mechanism for fine tuning of synaptic circuits.

Key words: cerebellum; chloride; GABA; imaging; parallel fibers; tonic inhibition

Significance Statement

This paper demonstrates that an unconventional form of signaling, known as tonic inhibition, is found in presynaptic terminals and affects conventional synaptic communication. Our results establish the basic characteristics and mechanisms of presynaptic tonic inhibition and show that it occurs in vivo as well as in isolated brain tissue.

Introduction

In addition to the well-established role of GABA in conventional, phasic synaptic inhibition, this neurotransmitter also participates in a tonic form of inhibition (Mody and Pearce, 2004; Farrant and Nusser, 2005). Tonic inhibition arises from constant activation of GABA_A receptors and has been studied most extensively in cerebellar granule cells, where it is the predominant form of inhibition (Kaneda et al., 1995; Brickley et al., 1996, 2001; Hamann et al., 2002; Rossi et al., 2003) and has been proposed to arise at least partially from channel-mediated release of GABA from glia (Lee et al., 2010). Although tonic inhibition has been studied mainly in slices, it is not a consequence of the slicing procedure because it has been observed in vivo in both cerebellar (Chadderton et al., 2004) and olfactory (Labarrera et al., 2013) granule cells. Tonic inhibition controls the gain and excitability of cerebellar granule cells (Mitchell and Silver, 2003), and at least some of the behav-

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ioral effects of ethanol consumption may arise from augmentation of tonic inhibition (Wallner et al., 2003; Hanchar et al., 2005).

Our current understanding of tonic inhibition is largely based on electrophysiological measurements of GABA-induced chloride currents. While such measurements offer very high sensitivity, they provide minimal spatial information. Thus, although it is clear that currents produced by tonic inhibition can be detected from recordings from the cell bodies of several types of neurons, it is rarely known which cellular compartments actually receive tonic inhibition. Here we use Clomeleon, an optogenetic fluorescent chloride indicator (Kuner and Augustine, 2000), to image the spatial extent of tonic inhibition of cerebellar granule neurons. We have taken advantage of transgenic mice that express Clomeleon in various subsets of neurons (Berglund et al., 2006); these mice have enabled imaging of local changes in intracellular chloride concentration ([Cl\(^-\)], associated with phasic synaptic inhibition (Berglund et al., 2006, 2008), heterogeneity of [Cl\(^-\)], and GABA responses in different compartments of retinal neurons (Duebel et al., 2006), and [Cl\(^-\)], increases in hippocampal neurons associated with both ischemia (Pond et al., 2006) and seizure activity (Glykys et al., 2009; Dzhala et al., 2010, 2012; Lillis et al., 2012; Glykys et al., 2014).

Our results follow-up on a preliminary report indicating that Clomeleon imaging allows visualization of tonic inhibition (Lee et al., 2010) and demonstrate that tonic inhibition approximately doubles resting [Cl\(^-\)], in cerebellar granule cells. Remarkably, tonic inhibition occurs not only on granule cell bodies (GCBs) and dendrites, but also on their axons, the parallel fibers (PFs). Tonic inhibition of PFs was observed in vivo and reduces both spontaneous and evoked release of glutamate from PF presynaptic terminals. Thus, presynaptic tonic inhibition may serve as an important regulator of information flow through the synaptic circuits of the cerebellar cortex. Given that somatic tonic inhibition is found throughout the brain, presynaptic tonic inhibition may also be a general mechanism for regulating neurotransmitter release during phasic synaptic transmission.

### Materials and Methods

#### Immunohistochemistry

Immunohistochemistry was used to determine the pattern of Clomeleon expression in the cerebellum. In brief, anesthetized CLM1 transgenic mice (Berglund et al., 2006; The Jackson Laboratory, RRID: IMSR_JAX:0136161) were transcardially perfused with phosphate-buffered saline (PBS) containing (in mM) as follows: 137 NaCl, 2.7 KCl, 10 NaH\(_2\)PO\(_4\), and 1.8 KH\(_2\)PO\(_4\) (pH 7.4 with HCl), then with a PBS solution containing 4% paraformaldehyde (PFA). Brain tissue was then removed and postfixed overnight in 4% PFA solution at 4°C. For subsequent cryoprotection, tissue was kept overnight in PBS solutions containing graded concentrations of sucrose ranging from 10%–30% (w/v). A freezing microtome postfixed overnight in 4% PFA solution at 4°C. For subsequent cryoprotection, brain sections were immunostained with the anti-YFP primary antibody, followed by anti-chicken Alexa-488 secondary antibody and then stained with DAPI (Invitrogen).

#### Calibration of Clomeleon

Clomeleonimmunofluorescent staining was performed as described by Berglund et al. (2006). For this purpose, an upright epifluorescence microscope (E600-FN, Nikon) was equipped with a 10 × 0.3 NA objective for the Clomeleon imaging experiments or with a 40 × 0.8 NA water-immersion objective for the electrophysiological experiments. This microscope included in its excitation pathway a mercury arc lamp, an electronic shutter (Uniblitz), an excitation filter (440 ± 10 nm), and a dichroic mirror (460 nm); the emission pathway included a filter wheel (Ludl Electronic Products) that alternated between two emission filters (485 ± 15 nm for CFP and 530 ± 15 nm for YFP; 71007A Cameleon 2, Chroma). Fluorescence excitation was produced by light pulses (200–500 duration, 0.5 Hz), and fluorescence emission was alternately collected at each wavelength with a back-illuminated, cooled CCD camera with on-chip multiplication gain control (Cascade 512B, Photometrics). Image acquisition was controlled by RatioTool software (See Imaging Systems) and a PowerMac G4 (Apple Computer). Further details of the imaging system can be found in Berglund et al. (2006).

To measure Clomeleon fluorescence in the granule cell and MLs, two ROIs covering these layers were defined. The intensity of YFP fluorescence, corrected for detector dark current and other sources of background signal, was divided by that of CFP to calculate a ratio in each ROI that is inversely proportional to [Cl\(^-\)] (Kuner and Augustine, 2000). Autofluorescence accounted for <1.5% of the total fluorescence and did not have any significant effect on the calculated ratio (Pond et al., 2006). Photobleaching (Berglund et al., 2005; Friedel et al., 2013) was minimized by using a low level of excitation light, with neutral density filters used to attenuate excitation light from the mercury lamp >500-fold. Because of the low levels of fluorescence emission under these conditions, pixels on the CCD camera chip were sometimes binned by a factor of 2 to increase signal/noise. If any obvious trend due to photobleaching remained, it was corrected by fitting a regression line or an exponential function to traces measured in the absence of responses.

For experiments where GABA or GABA receptor antagonists were locally applied (see Figs. 7, 8), images were produced by averaging raw images over 3 or 4 trials and smoothed by a Gaussian filter (20 μm) twice before calculating the fluorescence emission ratio. Such ratio images were then converted into [Cl\(^-\)]. All the image processing, analysis, and statistical tests were done by IgorPro 6.1 (WaveMetrics).

The measurements shown in Figures 2, 6, and 10 were done with 2-photon imaging, using either an Ultima IV microscope (Prairie Technologies) or an Olympus FV1000 microscope. Clomeleon was excited using 860 nm excitation light (Berglund et al., 2011) and either a 25 × or 40 × water-immersion objective (Olympus) with total output power <50 mW. A fluorescence filter cube with an emission beamsplitter (71007, Chroma) was used for fluorescence emission. During imaging, 512 × 512 pixel images were acquired continuously every 30 s, with 4 images averaged to yield the results shown.

#### Calibration of Clomeleon

The Clomeleon fluorescence emission ratio (YFP/CFP) was converted into [Cl\(^-\)] as detailed by Kuner and Augustine (2000) and Berglund et al. (2009). In brief, [Cl\(^-\)], was calculated based on the following equation:

\[
[\text{Cl}^-] = K_d \times (R_{\text{min}} - R)/(R - R_{\text{max}})
\]

where \(R\) is the measured emission ratio, \(R_{\text{min}}\) and \(R_{\text{max}}\) represent the values of \(R\) when Clomeleon is either Cl\(^-\) bound (\(R_{\text{min}}\)) or Cl\(^-\) free (\(R_{\text{max}}\)), and \(K_d\) is the effective Cl\(^-\) dissociation constant of Clomeleon (Grimley et al., 2013). The calibration constants (\(R_{\text{min}} = 0.85, R_{\text{max}} = 3.08, K_d = 144 \text{ mm}\)) for GCBS, and \(R_{\text{min}} = 0.82, R_{\text{max}} = 2.92, K_d = 175 \text{ mm}\) for PFs) were
determined in situ using solutions of F−, glutamate, and two known concentrations of CI− (50 and 134 mm), respectively, at pH 7.1. F− solution contained the following (in mM): 89 KF, 7 N-methyl-D-glucamine (NMDG), 7 HF, 1.25 NaH2PO4, 16 KHCO3, 20 v(+/−)-glutamate, 2 EGTA, and 5 NaOH. Glutamate solution contained the following (in mM): 45 NMDG, 45 N-gluconic acid, 92 K-gluconate, 13 KHCO3, 20 v(+/−)-glutamate, 2 EGTA, 5 NaOH, and 3.3 Mg-gluconate. 134 mm CI− solution contained the following (in mM): 45 NMDG, 45 HC1, 1.25 NaH2PO4, 15 KHCO3, 20 v(+/−)-glutamate, 2 EGTA, 5 NaOH, and 3.3 Mg-gluconate. 50 mm CI− solution was obtained by mixing appropriate proportions of glutamate and 134 mm CI− solution. The Cl−/OH− antipporter tributyltin acetate (20 μM) and the K+/H+ ionophore nigericin (20 μM) were first dissolved in ethanol and then diluted into solutions to remove the Cl− and H+/OH− gradients, respectively.

Measurement of intracellular pH. A stock solution of SNARF-5F AM ester (2 mM; Invitrogen) in Pluronic F-127 (20% w/v solution in DMSO; Invitrogen) was added to an oxygenated ACSF at a final concentration of 4–20 μM. After incubation at 36°C, cerebellar slices from C57BL/6 wild-type mice were exposed to the SNARF-5F AM solution for 30–60 min and washed in oxygenated ACSF for at least 15 min at room temperature. Slices loaded with SNARF-5F were then imaged as described above, except for an excitation filter (540.5 ± 12.5 nm), a dichroic mirror (565 μm cutoff), and emission filters (580 ± 12.5 and 630 ± 30 nm). SNARF-5F emission ratio (630/580) was calibrated in vitro, as described previously (Pond et al., 2006), by using the following equation:

\[
pH = K_d + \log \left( \frac{(R - R_{max})}{(R_{max} - R)} \right)
\]

where \( R \) is the measured 630/580 nm emission ratio, \( R_{max} \) and \( R_{min} \) represent the values when SNARF-5F is either H+ bound or H+ free, respectively, and \( K_d \) is the effective H+ dissociation constant of SNARF-5F.

The \( K_d \) of Clomeleon was then corrected for measured pH changes by using the following equation:

\[
K_d = K_d \times 10^{(pH - 7.1)}
\]

where \( K_d \) is CI− dissociation constant of Clomeleon obtained at pH 7.10 described above and \( K_d \) is pH-corrected CI− dissociation constant at any given pH (pH) measured by SNARF-5F.

In vivo imaging. Clomeleon transgenic mice (age 5–10 weeks) initially were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Their heads were shaved and the mice were then placed in a stereotaxic head frame. Isoflurane gas (2%) in oxygen was administered throughout the experiments, and the electrocardiogram was measured continuously to monitor the physiological status of the mice. Body temperature was measured by a rectal probe and maintained at 38°C by a feedback-controlled heating pad. Before making an incision, droplets of bupivacaine were applied subcutaneously. A wire mesh was attached to the skull with acrylic glue to provide an anchor point for dental acrylic resin, and a stainless steel head plate with a 2 mm opening was cemented above the craniotomy site. A craniotomy was performed above the cerebellum, and the dura was removed.

The GABAA receptor blocker SR95531 (SR; also called gabazine; Torris Bioscience) was administered either by topical application onto the surface of the cerebellum or via subcutaneous injection. For topical application, polyethylene tubing was filled with SR solution (2 μm in sterilized normal saline [0.9% w/v NaCl]) via suction and then connected to a Hamilton syringe in an injection pump. The tip of the tubing was filled with SR-free saline, which served as a control, and was placed at the edge of the craniotomical window. The time when solution switched between control and SR solutions (see Fig. 6E, F, horizontal bars) was determined in advance by filling the same tubing with a fluorescent dye solution of the same volume in the same manner. Alternatively, SR was administered by subcutaneous injection at a dose of 5 mg/kg body weight.

Regardless of the method of drug application, the imaging window was sealed with a glass coverslip (diameter: 5 mm; thickness: #1; Warner Instruments) and low-melting point agar (2%) in normal saline. The objective lens was immersed in a droplet of normal saline on the cover-slip. After completing the surgical procedures, isoflurane concentration was lowered to 0.5%–1% to maintain anesthesia during in vivo imaging.

Because solution exchange was practically impossible during in vivo experiments, the Clomeleon ratio was calibrated on the 2-photon microscope by using slices maintained at 38°C by a temperature controller (TC-344B, Warner Instruments). Although we were able to match the temperature, one of the critical determinants of Cl− binding to Clomeleon (Berglund et al., 2009), there may be other factors (e.g., circulating blood in the intact brain) and differential scattering of emission light in deeper tissue that affect CFP and YFP emission differently between in vivo experiments and calibration experiments. Thus, we think that absolute measurements of [Cl−], were more reliable in slice experiments than in vivo.

Electrophysiology. Conventional whole-cell patch-clamp recordings were obtained from Purkinje cells by an Axopatch 1D amplifier (Molecular Devices), as described previously (Takata et al., 2007). Pipettes (4–5 MΩ) were filled with a solution that contained (in mM): 130 K-gluconate, 2 NaCl, 0.5 EGTA, 20 HEPES, 4 Na2-ATP, 0.4 Na3-GTP, 4 MgCl2, pH was adjusted to 7.2 with KOH. The cells were voltage clamped at EC1 (approximately −70 mV) to isolate EPSCs.

The chamber was constantly superfused with oxygenated ACSF at 2 ml/min. Most experiments were done at room temperature (−24°C); in some cases, the slice was maintained at 34°C by an electronic temperature controller. ACSF containing SR (10 μM), TTX (Tocris Bioscience; 1 μM), bicuculline (Sigma; 20 μM), furosemide (Tocris Bioscience; 100 μM), GABA (Sigma; 10 μM–100 μM), CGP55845 (Tocris Bioscience; 2 or 50 μM), or acetazolamide (Sigma; 50 μM) was bath applied by diluting a stock solution into the ACSF before chamber superfusion. In the Ca2+-free ACSF solution, CaCl2 was replaced by an equimolar concentration of MgCl2 and EGTA (2 mM) was added to chelate remaining trace amounts of Ca2+. Following a solution change, measurements were made after reaching an apparent steady state. For the experiments shown in Figure 10C, there was a systematic shift in baseline [Cl−]; to measure responses to SR, this shift was subtracted by fitting a single exponential while masking the time of drug application.

To stimulate PFs, a glass electrode (tip diameter −10 μm) was used. This electrode was positioned in the ML near the Purkinje cell body and electrical stimuli (5–10 μA, 100–200 μs duration) were applied by a stimulator (S44, Grass Instruments) with a stimulus isolation unit (PSIU6, Grass Instruments). When examining synaptic facilitation with paired stimulation, stimulus pairs were repeated every 10 s.

Animal experiments. All animal procedures listed here were approved by the Duke University Animal Care and Use Committee or by the Institutional Animal Care and Use Committee of the Biopolis Biological Resource Center in Singapore.

Experimental measurements are expressed as mean ± SEM, unless indicated otherwise.

Results

Clomeleon expression in cerebellar granule cells

The CLM1 line reportedly expresses the optogenetic Cl− indicator, Clomeleon, in cerebellar granule cells (Berglund et al., 2006). In slices prepared from these mice, the cerebellar granule cell layer (GCL) was highly fluorescent and the molecular layer (ML) was more diffusely fluorescent, whereas no fluorescence was observed in the Purkinje cell layer (PCL) (Fig. 1A). This pattern of expression is consistent with Clomeleon being expressed in GCBs and dendrites (to yield fluorescence in the GCL) as well as in their axons, the PFs, within the ML.

To more precisely determine which cells express Clomeleon, we examined the spatial pattern of fluorescence within the cerebellum at higher magnification. Within the GCL, Clomeleon fluorescence appeared to be restricted to a dense population of neurons with small cell bodies (3–5 μm diameter), characteristic of granule cells (Fig. 1B, top). Occasional gaps in fluorescence apparently were caused by Golgi cells: labeling of Golgi cells, via an mGluR2 antibody (Fig. 1B, center), revealed that the nonfluor-
resent areas corresponded to Golgi cell bodies (Fig. 1B, bottom). More generally, there was minimal spatial overlap between the fluorescence signals from Clomeleon and mGluR2, indicating a lack of Clomeleon expression in Golgi cells. Similarly, within the ML, the diffuse pattern of Clomeleon fluorescence was associated with structures too small to be resolved (Fig. 1C, top), consistent with localization to sagitally sectioned PFs (which have a cross-sectional diameter of $<1 \mu m$). Clomeleon fluorescence was excluded from blood vessels as well as small spherical regions $\sim 10 \mu m$ in diameter. The spherical regions contained nuclei, as indicated by the nuclear label, DAPI (Fig. 1C, center). There was no overlap between Clomeleon and DAPI labeling within the ML (Fig. 1C, bottom); given that interneurons are the predominant cell type within the ML, we conclude that interneurons, both within the ML as well as the GCL, do not express Clomeleon. In summary, our results extend previous indications that granule cells are the only cells expressing Clomeleon within the cerebellar cortex of this mouse line (Berglund et al., 2006), allowing us to use this mouse to image $[Cl^-]$ in granule cells.

**Imaging tonic inhibition with Clomeleon**

By measuring the ratio of fluorescence emission of the CFP donor and YFP acceptor of Clomeleon, we could determine resting $[Cl^-]_i$ in both the GCBs within the GCL and their PFs within the ML (Fig. 2A, B). When measured at room temperature (24°C), resting $[Cl^-]_i$ was in the range of 5–10 mM in both compartments (Fig. 2C). On average, resting $[Cl^-]_i$ was slightly higher in PFs than in GCBs (two-tailed paired Student’s $t$-test; $t_{(23)} = 2.093; p = 0.048; n = 24$). From these values, we calculated the equilibrium
potential for \(\text{Cl}^-\) \(E_{\text{Cl}}\) to be −74 mV for GCBs and −65 mV for PFs. Thus, there is a substantial difference in \(E_{\text{Cl}}\) between GCBs and PFs. Very similar results were obtained when the measurements were repeated at a more physiological temperature (34°C; Fig. 2D): resting \([\text{Cl}^-]\) was significantly higher in PFs than in GCBs (two-tailed Wilcoxon paired-sample test; \(p = 0.016; n = 7\)).

Clomeleon also allowed us to image chloride-based inhibition of these cells. To visualize tonic inhibition caused by ambient GABA, we treated cerebellar slices with GABA\(_A\) receptor antagonists; in most experiments, we applied SR95531 (SR; also called gabazine; 10 \(\mu M\)), although occasionally we instead applied bicuculline (20 \(\mu M\)). Treatment with either antagonist reduced basal \([\text{Cl}^-]\), substantially (Fig. 3A), as expected if these drugs were blocking a constant \(\text{Cl}^-\) influx produced by tonic inhibition. Thus, Clomeleon imaging is capable of reporting the magnitude, time course, and spatial distribution of rises in \([\text{Cl}^-]\), produced by tonic inhibition.

The image shown in Figure 3A indicates that SR caused a reduction in \([\text{Cl}^-]\), both in the GCL, where the cell bodies and dendrites of the granule cells reside, as well as in the ML, where PF axons are the only Clomeleon-expressing structures. These reductions in \([\text{Cl}^-]\), were relatively rapid, reaching a peak within 1–2 min both in GCBs (Fig. 3B) and in PFs (Fig. 3C), and reversed promptly upon removal of the SR. This time course presumably reflects the competitive interaction between SR and ambient GABA, as well as the rate of drug addition/removal. At 24°C, the SR-induced decrease in \([\text{Cl}^-]\), was \(\sim 5 \text{ mM}\) and was very similar in magnitude in GCBs and PFs (Fig. 3D; two-tailed Wilcoxon paired-sample test; \(p = 0.054; n = 11\)). Significant reductions in \([\text{Cl}^-]\), were also observed in GCBs and PFs at a more physiological temperature (34°C), although these reductions were somewhat smaller (Fig. 3E; two-tailed Wilcoxon paired-sample test; \(p = 0.16; n = 7\)). These results indicate that tonic inhibition affects \([\text{Cl}^-]\), in PFs, in addition to the expected effects on \([\text{Cl}^-]\), in GCBs. In both granule cell compartments, tonic inhibition makes a very substantial contribution to \(\text{Cl}^-\) homeostasis and accounts for 25%–75% of the basal \([\text{Cl}^-]\).

Clomeleon is somewhat sensitive to protons, with lower pH increasing the \(\text{Cl}^-\) affinity of this indicator (Kuner and Augustine, 2000). Because we calibrated Clomeleon at a pH of 7.1, any deviation of intracellular pH from this value would result in an error when determining \([\text{Cl}^-]\), from the Clomeleon FRET ratio (Kuner and Augustine, 2000; Pond et al., 2006). We therefore measured intracellular pH in granule cells loaded with a pH indicator, SNARF-5F (Fig. 4A). The basal pH of GCBs and PFs was 7.15 ± 0.01 and 7.11 ± 0.02 (mean ± SEM; \(n = 12\)), respectively, which were statistically different (two-tailed Wilcoxon paired-sample test; \(p = 0.042; n = 12\)). During application of SR, pH in both compartments increased by 0.04 (Fig. 4B, C), and all relevant \([\text{Cl}^-]\), values described here have been adjusted accordingly (see Materials and Methods). However, in practice, this slight alkalization produced by SR application did not affect measured \([\text{Cl}^-]\), values appreciably because Clomeleon is virtually insensitive to pH at low \([\text{Cl}^-]\), (Kuner and Augustine, 2000). We therefore conclude that tonic inhibition actually elevates \([\text{Cl}^-]\), in addition to slightly acidifying pH.

Properties of tonic inhibition of presynaptic PFs

The remainder of our study largely focused on tonic inhibition of PFs, which presumably went undetected in previous electrophysiological measurements from GCBs. We next asked whether the properties of presynaptic tonic inhibition of PFs, as reported by Clomeleon imaging, are comparable with those identified for tonic inhibition of postsynaptic GCBs. For this purpose, we performed several pharmacological experiments that addressed the sources of GABA in the ML and the identity of the GABAA receptors mediating the response of PFs to this GABA.

Tonic inhibition of cerebellar GCBs is mediated by ambient release of GABA that does not require electrical activity in presynaptic neurons (Rossi et al., 2003). To determine whether tonic inhibition of PFs exhibits similar characteristics, we examined the effects of TTX. Unlike bicuculline or SR, TTX (1 \(\mu M\)) had no significant effect on basal \([\text{Cl}^-]\), (Fig. 5A); TTX produced a reduction of 0.5 ± 0.4 mM (mean ± SEM; \(n = 6\)) in basal \([\text{Cl}^-]\), providing an indication that tonic inhibition of PFs does not require neuronal activity. More importantly, bicuculline (20 \(\mu M\))
still blocked tonic inhibition in the presence of TTX; the changes in $[\text{Cl}^-]$ produced by bicuculline were similar (two-tailed Wilcoxon paired-sample test; $p = 0.22$; $n = 6$) in the absence or presence of TTX (Fig. 5A,B). This provides a second indication that tonic inhibition of PFs does not require neuronal activity. Similar results were observed in GCBs as well; bicuculline decreased $[\text{Cl}^-]$, similarly in the absence or presence of TTX (2.1 ± 0.5 mM vs 1.7 ± 0.4 mM; mean ± SEM; $n = 6$; two-tailed Wilcoxon paired-sample test; $p = 0.22$), yielding one further indication that tonic inhibition of granule cells does not require neuronal activity.

Tonic inhibition of cerebellar GCBs also does not require Ca$^{2+}$-dependent exocytosis (Rossi et al., 2003). To determine whether this was true for tonic inhibition of PFs, we examined the effect of removing Ca$^{2+}$ from the extracellular solution. Treatment with Ca$^{2+}$-free solution caused an increase in basal $[\text{Cl}^-]$, (Fig. 5C), yielding a mean increase in $[\text{Cl}^-]$, of 1.5 ± 0.6 mM (mean ± SEM; $n = 9$). Although the reason for this increase is not clear, it is the opposite of what would be expected if tonic inhibition of PFs depended upon Ca$^{2+}$. Further, this rise in $[\text{Cl}^-]$, reduces the driving force on $\text{Cl}^-$ movement which, in turn, would be expected to reduce the $\text{Cl}^-$ fluxes associated with tonic inhibition. Nonetheless, SR still decreased $[\text{Cl}^-]$, in the absence of Ca$^{2+}$ (Fig. 5C), although, as expected, the effect of SR was significantly smaller in Ca$^{2+}$-free solution than in control conditions (Fig. 5D; two-tailed Wilcoxon paired-sample test; $p = 0.04$; $n = 9$). Similar results were observed in GCBs as well (5.0 ± 1.0 mM in control vs 2.3 ± 0.5 mM in Ca$^{2+}$-free; mean ± SEM; $n = 9$; two-tailed Wilcoxon paired-sample test; $p = 0.004$). These results indicate that tonic inhibition of presynaptic PFs reported by Clomeleon imaging is due to tonic release of GABA via a mechanism that is independent of neuronal activity and does not depend entirely upon calcium influx, as has been reported previously for tonic inhibition of GCBs. These parallels between tonic inhibition of PFs and GCBs are consistent with the proposal that sustained, channel-mediated release of GABA from glia is the source of ambient GABA responsible for both forms of tonic inhibition (Lee et al., 2010).

Figure 4. pH measurements in the cerebellar granule cells. A, A SNARF-5F-loaded cerebellar slice. Emission at 630 nm. B, Global alkalization in the cerebellar cortex was seen when SR (10 mM) was applied in the bath. A part of the cerebellar cortex was imaged with the 40 × objective lens. ML interneurons were loaded as well (arrows). Note the lack of SNARF-5F loading in Purkinje cells (*). Image was integrated over the last minute of SR application. C, Time courses of intracellular pH changes following SR application in the two compartments of granule cells, GCBs and PFs. Traces represent averages of 12 experiments.

Figure 5. Properties of tonic inhibition of PFs. A, TTX (1 μM) did not change basal $[\text{Cl}^-]$, in PFs or block the changes in $[\text{Cl}^-]$, produced by bicuculline (Bic; 20 μM). B, Mean changes in resting $[\text{Cl}^-]$, of PFs produced by application of bicuculline alone (Bic) or by bicuculline in the presence of TTX (Bic TTX; $n = 6$). C, Treatment of cerebellar slices with Ca$^{2+}$-free extracellular solution increased basal $[\text{Cl}^-]$, in PFs but did not block the response of PFs to SR (10 μM). D, Mean changes in resting $[\text{Cl}^-]$, of PFs produced by application of SR in normal saline (SR) and in the absence of Ca$^{2+}$ (SR 0 Ca; $n = 9$). * $p < 0.05$ (Wilcoxon test). E, $[\text{Cl}^-]$, of PFs was reduced by SR as well as by furosemide (Furo; 100 μM). F, Mean changes in resting $[\text{Cl}^-]$, produced by application of SR or furosemide ($n = 7$).
structure of the cerebellum (~200 μm below the pial surface (Fig. 6B)). Such images were strikingly similar to what was observed in cerebellar slices in vitro (Fig. 2A).

Unlike in slice preparations, there was no significant difference in resting [Cl⁻] between PFs and GCBs (7.5 ± 2.5 mM and 10.0 ± 1.7 mM, respectively; n = 7; two-tailed Wilcoxon paired-sample test; p = 0.47).

Figure 6C, D shows representative changes in [Cl⁻], in the GCBs and PFs, respectively, following subcutaneous injection of SR (5 mg/kg body weight) (Nelson et al., 2002). In these experiments, [Cl⁻], started dropping ~5 min after injection of the SR solution and continued to decrease for the next 10 min or so. Presumably, the slow time course of these responses reflects the time required for SR to reach the cerebellum. Similar observations were made in a total of 4 animals.

We also applied SR (2 mM) topically onto the surface of the cerebellum in a different set of animals (n = 3). To apply SR topically, a tube was placed at the edge of the craniotomy, sealed with agar and a coverslip (Fig. 6A). The tubing was connected to a syringe pump which continuously superfused solutions during an imaging session, initially delivering normal saline as a control and then switching to SR-containing saline. Although there was again a decline in [Cl⁻], in GCBs (Fig. 6E) and in PFs (Fig. 6F) during treatment with SR, as observed during subcutaneous application, these decreases were preceded by an apparent increase in [Cl⁻]. The initial response was immediate and much faster than the time required for SR to diffuse from the pial surface to the plane of focus where Clomeleon fluorescence was imaged. We suspect that this initial effect was an artifact caused by the concentrated SR solution, which was bright yellow and could differentially absorb Clomeleon emission. Considering the drops in [Cl⁻], observed with both application methods, the magnitude of this SR-induced drop in [Cl⁻], was similar in GCBs and in PFs (Fig. 6G; two-tailed Wilcoxon paired-sample test; p = 0.94; n = 7), consistent with our observations in slices (Fig. 3E). Thus, we conclude that tonic inhibition of PFs occurs both in slices and in vivo. Our results also confirm a previous electrophysiological demonstration of tonic inhibition of GCBs in vivo (Chaderton et al., 2004). Because these measurements were made in adult mice (age 5–10 weeks), we can conclude that tonic inhibition occurs in adult mice as well as in the younger mice used for our slice experiments.

In summary, Clomeleon imaging allowed us to monitor tonic inhibition of PFs both in vitro and in vivo. This approach revealed that tonic inhibition of PFs shares most of the properties of tonic inhibition of GCBs observed in previous electrophysiological studies.

Tonic inhibition of presynaptic PFs

Because tonic inhibition previously had been detected only with electrical recordings from GCBs, it is possible that tonic inhibition only occurs in the somatodendritic region of these cells. In contrast, tonic inhibition of PFs was also revealed by Clomeleon imaging in vitro, where the somatodendritic region is not available for study. In vivo, the somatodendritic region of PFs is not accessible to patch-clamp recordings, yet the same drop in intracellular chloride was observed in vivo with two-photon imaging and Clomeleon transgenic mice. Thus, tonic inhibition of PFs functions as a release-inhibitory mechanism that occurs both in vivo and in vitro.

The GABA_A receptors involved in tonic inhibition of GCBs contain α6 subunits (Hamann et al., 2002), which are preferentially blocked by low concentrations of furosemide (Wall, 2002). To determine whether such receptors are involved in tonic inhibition of PFs, we asked whether the reduction of [Cl⁻], in PFs produced by SR was mimicked by furosemide (100 μM). We found that furosemide also reduced [Cl⁻], in PFs (Fig. 5E). The changes in [Cl⁻], produced by furosemide were very similar in magnitude to those produced by SR (Fig. 5F), with no significant difference between the mean changes in [Cl⁻], produced by the two drugs (two-tailed Wilcoxon paired-sample test; p = 0.81; n = 7). Similar results were observed for GCBs: there was no significant difference between the mean changes in [Cl⁻], produced by the two drugs (3.6 ± 0.8 mM with SR vs 3.6 ± 0.4 mM with furosemide; n = 7; two-tailed Wilcoxon paired-sample test; p = 0.94). These results indicate that the high basal [Cl⁻], of PFs is due to the tonic activation of high-affinity GABA_A receptors that contain α6 subunits. Further, we can conclude that the changes in basal [Cl⁻], produced by blockade of these GABA_A receptors are due to reduction of this tonic inhibition.

Tonic inhibition of PFs in vivo

To determine whether tonic inhibition of PFs occurs in vivo, we used 2-photon imaging to measure [Cl⁻], in the intact brain of Clomeleon transgenic mice. For this purpose, a window was made in the skull of mice anesthetized by isoflurane, and Clomeleon fluorescence was imaged in granule cells in the fourth and fifth lobule or the sixth lobule of the cerebellum, near the midline (Fig. 6A). In these conditions, we could visualize the laminar
this case, the decrease in $[\text{Cl}^-]$ of PFs produced by application of SR (Figs. 3, 5, and 6) would be an indirect consequence of blocking tonic inhibition in the somata and dendrites of granule cells, with $\text{Cl}^-$ diffusing from PFs to the rest of the granule cell (Fig. 7A, left). Alternatively, the drop in PF $[\text{Cl}^-]$ could result directly from tonic inhibition of PFs (Fig. 7A, right). Local application of SR provides a means of distinguishing between these two models. If $[\text{Cl}^-]$ changes in PFs are caused by intracellular $\text{Cl}^-$ diffusion, then these changes should not occur when SR is locally applied onto PFs. In contrast, if functional GABA$_A$ receptors are present on PFs, then $[\text{Cl}^-]$ in PFs should be reduced in response to local application of SR.

To distinguish between these two possibilities, we locally applied SR in cerebellar slices. Local application of SR onto GCBs caused a localized decrease in $[\text{Cl}^-]$ (Fig. 7B), with the mean decrease in $[\text{Cl}^-]$ in GCBs being $1.9 \pm 0.4$ mM (mean $\pm$ SEM; $n = 7$). This response was transient and recovered soon after the 10-s-long application of SR ended (Fig. 7C), presumably due to rapid diffusion of the drug. This result is predicted by both of the models shown in Figure 7A and serves as a positive control to indicate that local SR application is effective in blocking tonic inhibition. To distinguish between the two models, we next locally applied SR onto PFs. This caused a transient decrease in $[\text{Cl}^-]$, in PFs (Fig. 7D, E). The mean decrease in $[\text{Cl}^-]$ in PFs was $2.0 \pm 0.3$ mM (mean $\pm$ SEM; $n = 7$). This result is consistent with the model shown in Figure 7A (right) and therefore indicates a tonic inhibition of PFs via sustained activation of GABA$_A$ receptors that reside on PFs.

**Biphasic GABA actions on PFs**

Although our results with GABA$_A$ receptor antagonists indicate that GABA tonically inhibits PFs, local application of GABA or GABA$_A$ agonists reportedly excites PFs (Stell et al., 2007; Stell, 2011; Astorga et al., 2015). To reconcile these seemingly contradictory results, we locally applied GABA (1 mM) onto PFs. Local application of GABA onto PFs decreased $[\text{Cl}^-]$ (Fig. 8A, B). These responses to GABA were reduced significantly by SR (100 mM; from $0.73 \pm 0.14$ mM to $0.10 \pm 0.05$ mM; mean $\pm$ SEM; $n = 11$; two-tailed Wilcoxon paired-sample test; $p = 0.001$), but not by a GABA$_B$ antagonist, CGP55845 (2–50 nM; from $2.5 \pm 0.7$ mM to $2.5 \pm 0.6$ mM; mean $\pm$ SEM; $n = 7$; two-tailed Wilcoxon paired-sample test; $p = 0.38$). This indicates that the GABA-induced decrease in $[\text{Cl}^-]$, is mediated solely by GABA$_A$ receptors. This decrease in $[\text{Cl}^-]$ must result from an efflux of $\text{Cl}^-$ that would depolarize the PF membrane potential, indicating that GABA is excitatory under these conditions. This confirms previous conclusions (Stell et al., 2007; Stell, 2011; Astorga et al., 2015).

In contrast, local application of GABA onto GCBs increased $[\text{Cl}^-]$ (Fig. 8C–D; $0.7 \pm 0.2$ mM; mean $\pm$ SEM; $n = 10$), indicating an inhibitory action of GABA on this compartment of the granule cell. Further, these results indicate limited diffusion of $\text{Cl}^-$ between the PFs and GCBs.

The concentration of ambient GABA that produces tonic inhibition has been estimated to be $\approx 160$ nM (Santhakumar et al., 2006; Lee et al., 2010), which is much lower than the concentration produced by local GABA application (1 mM in our experiments). Thus, the simplest explanation for our results showing that both SR and high concentrations of GABA lower $[\text{Cl}^-]$, in PFs is that GABA has different actions at different concentrations. To test this hypothesis, we bath-applied GABA at various concentrations while measuring $[\text{Cl}^-]$. Treating PFs with 100 nM GABA, near the concentration thought to mediate tonic inhibition, increased $[\text{Cl}^-]$ (Fig. 9A). This is consistent with the observed reduction in $[\text{Cl}^-]$, caused by SR blockade of tonic inhibition (Fig. 3). Higher concentrations of GABA caused larger increases in $[\text{Cl}^-]$, with maximal effects observed at 1 μM GABA (Fig. 9B, C); 10 μM GABA caused an increase in $[\text{Cl}^-]$ in PFs similar to that produced by 1 μM GABA (Fig. 9C). However, 100 μM GABA produced a smaller increase than that produced by lower concentrations of GABA; in some cases, 100 μM GABA caused a small net decrease in $[\text{Cl}^-]$ (Fig. 9D), revealing biphasic effects of GABA on PFs (Fig. 9E; $n = 8$). This biphasic property accounts for the decreases in $[\text{Cl}^-]$ observed both when blocking tonic inhibition of PFs (Fig. 3) and when locally applying 1 mM GABA onto PFs (Fig. 8). In contrast, increasing GABA concentrations produced only monotonic rises in $[\text{Cl}^-]$ in GCBs (Fig. 9F; $n = 8$).

The dose–response curve for GCBs was well fit by the Hill equation (Fig. 9F, curve), with a Hill coefficient of 0.66 and a $K_D$ of 160 nM ($n = 8$). A similar relationship could also describe the responses of PFs to GABA concentrations of $\approx 10$
μM (Fig. 9E, curve; a Hill coefficient of 0.73 and a $K_d$ of 13 nM; $n = 8$), where the actions of GABA to decrease $[\text{Cl}^-]_i$ were not evident. The submicromolar $K_d$ for both responses is consistent with the very high affinity characteristic of GABA$_A$ receptors involved in tonic inhibition (Quirk et al., 1994; Saxena and Macdonald, 1996; Rossi and Hamann, 1998).

The two processes underlying the biphasic response of PFs to GABA could be dissociated by treatment with furosemide (100 μM). This drug completely prevented GABA from elevating $[\text{Cl}^-]_i$ in PFs (Fig. 9E; $n = 7$). This blockage of GABA responses was statistically significant (two-way ANOVA; the main effect of furosemide: $F_{(1,78)} = 46.1, p < 0.05$; the main effect of GABA: $F_{(1,78)} = 2.38, p < 0.05$; their interactions: $F_{(5,78)} = 2.36, p < 0.05$; in Dunnett’s pairwise comparison, all the furosemide conditions were significantly different from control, $p < 0.05$ and control responses to 1 and 10 μM GABA were significantly different from 0, $p < 0.05$). The same was true in GCBs (Fig. 9F; $n = 7$; two-way ANOVA; the main effect of furosemide: $F_{(1,78)} = 52.9, p < 0.05$; the main effect of GABA: $F_{(1,78)} = 5.01, p < 0.05$; their interactions: $F_{(5,78)} = 3.21, p < 0.05$; in Dunnett’s pairwise comparison, all the furosemide conditions were significantly different from control, $p < 0.05$ and control responses to 1, 10, and 100 μM GABA were significantly different from 0, $p < 0.05$). This suggests that exogenous GABA raises $[\text{Cl}^-]_i$ by activating high-affinity GABA$_A$ receptors that contain $\alpha_1$ subunits (Wall, 2002). In contrast, the reduction of $[\text{Cl}^-]_i$ produced in PFs by higher doses of GABA was not blocked by furosemide (Fig. 9E), including when 1 mM GABA was locally applied onto PFs with the procedure illustrated in Figure 8 (data not shown). These results indicate that the ability of high concentrations of GABA to cause Cl$^-$ efflux is due to a different population of GABA$_A$ receptors with a very low affinity for GABA. This affinity must be >100 μM because at this concentration the dose–response curve measured in the presence of furosemide shows no sign of saturation (Fig. 9E). This is consistent with previous work showing that phasic inhibition of granule cells is mediated by low-affinity synaptic GABA$_A$ receptors that are insensitive to furosemide (Rossi and Hamann, 1998; Hamann et al., 2002).

Anion fluxes underlying GABA inhibition and excitation of PFs

How can different concentrations of GABA cause Cl$^-$ to flow in opposite directions? The efflux of Cl$^-$ during the action of high [GABA] indicates that the PF membrane potential is more negative than $E_{\text{Cl}}$ (−65 mV for PFs). This arises from the high resting $[\text{Cl}^-]_i$ of PFs (Fig. 2C,D), as well as the likely contribution of a relatively high resting $K^+$ conductance to maintain a hyperpolarized resting membrane potential. However, the action of low [GABA] to create a sustained influx of Cl$^-$ requires an additional depolarizing drive to maintain the membrane potential at a level more positive than $E_{\text{Cl}}$; otherwise, the resting membrane potential would quickly hyperpolarize to $E_{\text{Cl}}$ and Cl$^-$ influx would cease. It is known that sustained activation of GABA$_A$ receptors can depolarize neurons because efflux of HCO$_3^-$ through GABA receptors occurs even when the electrochemical gradient for Cl$^-$ has collapsed (Staley et al., 1995; Staley and Proctor, 1999; Ferrini et al., 2013). We therefore next considered the possible role of HCO$_3^-$ in maintaining the driving force for Cl$^-$ influx during tonic inhibition.

The contribution of HCO$_3^-$ to tonic inhibition was examined by measuring Cl$^-$ fluxes while removing HCO$_3^-$ (Fig. 10A,B). To eliminate HCO$_3^-$, NaHCO$_3$ in the extracellular solution was replaced by HEPES and CO$_2$ was replaced with O$_2$; extracellular pH was maintained at 7.4. In addition, intracellular generation of HCO$_3^-$ by carbonyl anhydrase was blocked by the inhibitor acetazolamide (50 μM) (Kim and Trussell, 2009; Ferrini et al., 2013). Such depletion of intracellular HCO$_3^-$ could be observed as an alkalization of cytoplasmic pH in PFs (Fig. 10A,B). This effect upon presynaptic pH was statistically significant ($n = 3$; one-way ANOVA; $F_{(2,6)} = 12.8; p = 0.007$; followed by Dunnett’s test; $q(6, 3) = 3.33; p = 0.028$). Removal of HCO$_3^-$ also reduced resting $[\text{Cl}^-]_i$ significantly (Fig. 10C,D; $n = 7$; one-way ANOVA; $F_{(1,18)} = 13.7; p = 0.0002$; followed by Dunnett’s test; $q(18, 3) = 4.31; p = 0.0008$). This effect perhaps was due to the predicted reduction in driving force for Cl$^-$ influx during tonic inhibition. More importantly, the absence of HCO$_3^-$ caused a complete loss of the drop in $[\text{Cl}^-]$, caused by SR blockade of tonic inhibition (Fig. 10E); this effect was statistically significant ($n = 4$; $p < 0.05$; one-way ANOVA followed by Dunnett’s test). Further, this effect on SR responses reversed when HCO$_3^-$ was restored, indicating that it is not a nonspecific effect associated with HCO$_3^-$ removal. Together, these results indicate that HCO$_3^-$ is required to sustain the driving force for Cl$^-$ influx during tonic inhibition.
In conclusion, PFs possess at least two types of GABA$_A$ receptors (Fig. 11). One type of GABA$_A$ receptor has a high affinity for GABA and is tonically activated by ambient GABA, yielding an influx of Cl$^-$ that produces tonic inhibition (Fig. 11, left). HCO$_3^-$ permeation in these receptors apparently is significant and required to sustain tonic inhibition by providing the driving force for Cl$^-$ influx. These receptors are blocked by furosemide (Figs. 5E,F, 9E). The second type of GABA$_A$ receptor has a low affinity for GABA (Fig. 11, right). Activation of this receptor by high concentrations of GABA ($\geq 50 \mu M$) causes Cl$^-$ efflux that can depolarize PFs, evident as both an enhancement of PF transmission (Stell et al., 2007; Pugh and Jahr, 2011; Dellal et al., 2012; Astorga et al., 2015) and a decline in [Cl$^-$/HCO$_3^-$], (Figs. 8B, 9D). These receptors are not blocked by furosemide (Fig. 9E) and apparently are not permeable to HCO$_3^-$; the driving force for Cl$^-$ efflux through these receptors is provided by a hyperpolarized resting membrane potential, due to resting K$^+$ efflux, as well as possible desensitization of the high-affinity, HCO$_3^-$/Cl$^-$ permeable GABA receptors.

Regulation of glutamate release by presynaptic tonic inhibition

Tonic inhibition of presynaptic PFs by ambient GABA could also regulate the amount of transmitter released from PF terminals, thereby influencing phasic synaptic transmission between granule cells and postsynaptic Purkinje cells. To examine this possibility, we used SR application to block tonic inhibition while measuring EPSCs produced in Purkinje cells in response to glutamate released by PFs. In these experiments, we held the Purkinje cells at E$_{K,L}$ to avoid possible complications caused by SR blocking IPSCs generated in Purkinje cells by phasic release of GABA from interneurons (Komnenth et al., 1990; Llano et al., 1991; Vincent and Marty, 1996; Kim et al., 2014). These experiments were done in sagittal slices; in such slices, PFs are severed from GCBs during the slicing procedure, thereby eliminating any possible contribution of GCBs to measured synaptic responses.

We first examined the effect of SR on spontaneous release of glutamate from PF terminals. Small spontaneous inward currents could be detected in Purkinje cells (Fig. 12A). Treatment with the glutamate receptor antagonist, kynurenic acid (3 mM), reduced the frequency of these currents by 99%, from 5.4 ± 1.4 Hz to 0.1 ± 0.1 Hz (mean ± SEM; n = 6 cells). This indicates that these currents represent glutamatergic EPSCs that were virtually uncontaminated by GABAergic IPSCs and presumably reflect spontaneous miniature EPSCs (Bordey and Sontheimer, 2003). Blockade of tonic inhibition, by application of SR, caused the frequency of these spontaneous EPSCs to increase (Fig. 12A).

This effect of SR treatment was relatively rapid, reaching a peak within 1–2 min after beginning SR superfusion (Fig. 12B). This approximately parallels the time course of changes in [Cl$^-$/HCO$_3^-$], caused in PFs by SR (Fig. 3C). SR increased the mean frequency of spontaneous EPSCs by 172 ± 70% (mean ± SEM; n = 6 cells) over the values measured before SR treatment (Fig. 12C). This increase in EPSC frequency was significant (two-tailed Wilcoxon paired-sample test; p = 0.03; n = 6 cells). In contrast, SR had no effect on the distribution of spontaneous EPSC amplitudes (Fig. 12D); Kolmogorov–Smirnov test; D = 0.014; p = 0.13; n [weighted number of elements] = 7,072) or the mean amplitude of spontaneous EPSCs (Fig. 12E; two-tailed Wilcoxon paired-sample test; p = 0.56; n = 6 cells), indicating a lack of effect of SR treatment on the properties of postsynaptic Purkinje cells (Fatt and Katz, 1952). Thus, spontaneous release of glutamate from presynaptic PFs is limited by tonic inhibition.

Figure 10.

Two types of GABAₐ receptors in PFs. High-affinity receptors are permeable to HCO₃⁻, with the efflux of this anion providing a positive driving force for Cl⁻ that enables persistent influx of Cl⁻ through these receptors (left). Low-affinity receptors are not permeable to HCO₃⁻, reversing the polarity of the electrochemical driving force for Cl⁻ at high GABA levels (right).

We next examined the effect of SR on EPSCs evoked by PF stimulation. In these experiments, an extracellular stimulating electrode was used to activate presynaptic PFs and was placed near the Purkinje cell body to minimize possible shunting of the evoked EPSCs along Purkinje cell dendrites by GABAergic inhibition. The inward currents evoked by such stimuli were almost completely blocked by the combined application of the glutamate antagonists kainic acid (3 mM) and CNQX (100 µM; Fig. 13A; n = 10), indicating that these currents were almost purely glutamatergic EPSCs.

SR treatment increased the amplitude of these evoked EPSCs (Fig. 13A). The effect of SR on EPSC amplitude was reversible (Fig. 13B,C). On average, SR treatment increased EPSC amplitude by 16 ± 8% (mean ± SEM; n = 9; Fig. 13C), and this effect was statistically significant (two-tailed Wilcoxon paired-sample test; p = 0.037). These results indicate that tonic inhibition suppresses phasic transmission between presynaptic PFs and postsynaptic Purkinje cells. Given the results above indicating that SR does not alter the amplitude of spontaneous EPSCs (Fig. 12D,E), we conclude that this effect of SR on EPSC amplitude is due to tonic inhibition reducing the amount of glutamate released from presynaptic PFs in response to action potentials (Del Castillo and Katz, 1954).

To provide further support for our conclusion that SR affects phasic synaptic transmission by acting on presynaptic PFs, we next examined the effects of SR on synaptic facilitation. Given that facilitation is very sensitive to the probability of transmitter release, changes in transmitter release from presynaptic terminals usually alter the degree of facilitation (Dobrunz and Stevens, 1997; Oleskevich et al., 2000). Under control conditions, EPSCs facilitated when pairs of PF stimuli were applied at an interval of 100 ms, as has been described previously (Konnerth et al., 1990; Atluri and Regehr, 1996; Kreitzer and Regehr, 2000). Application of SR caused the second EPSC to show less facilitation than in control conditions. This effect on synaptic facilitation was readily observed after normalizing the amplitudes of EPSCs produced by the first stimuli (Fig. 13D). The effect of SR on facilitation was reversible and statistically significant (Fig. 13E; two-tailed Wilcoxon paired-sample test; p = 0.02; n = 9). This reduction in facilitation provides a second indication that tonic inhibition normally acts to decrease the probability of glutamate release from presynaptic terminals of PFs in response to action potentials.

Finally, we examined a third index of presynaptic function, EPSC coefficient of variation (CV) (Del Castillo and Katz, 1954; Silver et al., 1996; Silver et al., 1998; Sola et al., 2004). This parameter also was reversibly reduced by SR, and the effect of SR on the coefficient of variation was statistically significant (Fig. 13F; two-tailed Wilcoxon paired-sample test; p = 0.002; n = 10). The decrease in the CV produced by SR is a third independent indication that tonic inhibition reduces the probability of glutamate release from presynaptic PFs in response to action potentials.

In conclusion, tonic inhibition reduces both spontaneous and evoked release of glutamate from PFs and thereby limits phasic synaptic transmission between PFs and Purkinje cells.

Discussion

We used Clomeleon imaging to detect tonic inhibition of cerebellar granule cells. Tonic inhibition was not limited to GCBs and dendrites: granule cell axons, the PFs, also have tonically activated GABAₐ receptors. The properties of PF tonic inhibition are much like those of tonic inhibition of GCBs, with the important additional property of regulating neurotransmitter release from PF presynaptic terminals.

Optogenetic imaging of tonic inhibition

Optogenetic techniques are revolutionizing our ability to both control and monitor neuronal activity, as well as to map neural
circuits (Baker et al., 2008; Berglund et al., 2008; Mancuso et al., 2011; Deisseroth, 2015). Here we have used the optogenetic indicator, Clomeleon (Kuner and Augustine, 2000), to monitor tonic inhibition. Transgenic mice that express Clomeleon in cerebellar granule cells allowed us to visualize tonic inhibition of these cells, both in vitro and in vivo. This approach revealed that tonic inhibition occurs in PFS, a location that has gone undetected in previous electrophysiological studies. Thus, Clomeleon imaging provides a means of detecting tonic inhibition offering technical ease, spatial resolution, and targeting to genetically defined neuronal populations. This approach should greatly promote efforts to identify the locations and physiological significance of tonic inhibition throughout the brain, as well as to understand the underlying mechanisms.

Presynaptic GABA<sub>α</sub> receptors

Presynaptic inhibition of phasic synaptic transmission by GABA<sub>α</sub> receptors was first identified in the crayfish neuromuscular junction (Dudel and Kuffler, 1961) and subsequently has been found in many types of synapses in the mammalian CNS (for review, see Trigo et al., 2008). Our work adds cerebellar PFS to the list of presynaptic terminals that evince inhibition mediated by GABA<sub>α</sub> receptors. Thus, GABA<sub>α</sub> receptors join adenosine and GABA<sub>B</sub> receptors (Dittman and Regehr, 1996, 1997) as presynaptic modulators of excitatory transmission in the cerebellar ML.

Following blockade of tonic inhibition, we observed a substantial increase in spontaneous excitatory synaptic transmission between granule cells and Purkinje cells (Fig. 12) and a smaller but significant enhancement of evoked excitatory transmission (Fig. 13). These experiments were made in sagittal slices, where the vast majority of granule-cell axons (PFS) were severed from their cell bodies in the granular layer, thus eliminating any contribution of granule-cell dendrites and cell bodies to the response to SR application. Still, SR can block GABA<sub>α</sub> receptors on Purkinje cell dendrites, and this could affect propagation of synaptic current along the dendrites via shunting inhibition, potentially contributing to changes in EPSCs recorded in the cell body. However, this cannot explain our results because there was no observable change in the amplitude of spontaneous EPSCs during SR treatment (Fig. 12 D,E). Further, we also observed changes in synaptic facilitation and coefficient of variance, measures that solely depend upon presynaptic properties. Together, we can conclude that tonic inhibition reduces both spontaneous and evoked release of glutamate from PFS.

The magnitude of the effect of presynaptic tonic inhibition on PF synaptic transmission (~16%) was smaller than some other forms of synaptic plasticity found at this synapse: long-term potentiation can strengthen PF synapses on Purkinje cells by up to 75% (Hirano, 1990), and long-term depression can reduce transmission at these synapses by as much as 50% (Tanaka et al., 2007). However, given that ~150 PF synapses must be active to fire an action potential in a Purkinje cell (Isobe and Barbour, 2002), a 16% inhibition of PF synaptic transmission will negate the effect of >20 PF inputs to a Purkinje cell. This would be expected to significantly alter integration of PF inputs by Purkinje cells. The substantial effects of tonic inhibition on synaptic facilitation (Fig. 13 E) and synaptic variability (Fig. 13 F) would be expected to have further dynamic effects on integration of PF synaptic input.

Our conclusion that presynaptic GABA<sub>α</sub> receptors control phasic synaptic transmission between cerebellar granule cells and Purkinje cells is consistent with previous results. Immunoelectron microscopy studies have identified GABA<sub>α</sub> receptors on PF terminals (Stell et al., 2007). In cerebellar granule cells, single electrical stimuli can generate repetitive firing of action potentials that is blocked by muscimol, a GABA<sub>α</sub> agonist (Isobe et al., 2004). Further, both spontaneous and evoked glutamate release from PFS is regulated by activation of GABA<sub>α</sub> receptors (Stell et al., 2007; Stell, 2011; Astorga et al., 2015).

One apparent discrepancy is that application of GABA<sub>α</sub> agonists enhances glutamate release from PFS (Stell et al., 2007), although we have found that ambient GABA is inhibitory. We have demonstrated that this difference is due to the GABA concentrations present in the two conditions. Specifically, during tonic inhibition, ambient GABA concentrations are in the submicromolar range (Santhakumar et al., 2006), a concentration where GABA increases [Cl<sup>-</sup>] in PFS (Fig. 9 F). In contrast, the excitatory actions of GABA on PFS require high GABA concentrations. Thus, in addition to its tonic inhibitory action, high concentrations of GABA (50 µM to 1 mM) cause a Cl<sup>-</sup> efflux (Figs. 8, 9) that depolarizes PFS (Astorga et al., 2015), accounting for the observation that activation of GABA<sub>α</sub> receptors by such
concentrations of GABA can augment glutamate release (Stell et al., 2007). The timing of GABA application also appears to be important for inhibitory action of GABA. Stell (2011) reported that the effect of the GABA_\text{A} agonist, muscimol, on action potential firing of PFs became inhibitory when application was prolonged, consistent with our observation that the sustained presence of ambient GABA is inhibitory.

Manipulation of HCO_3^- showed that this anion enables a sustained influx of Cl^- during tonic inhibition (Fig. 10). Although HCO_3^- supports tonic inhibition, such an effect would prevent Cl^- efflux during the excitatory action of GABA. We thus hypothesize that differences in HCO_3^- permeability between the high- and low-affinity GABA_\text{A} receptors of PFs underlies the bi-phasic nature of GABA action (Figs. 8-10). Differences have been observed in the HCO_3^- permeability of GABA receptors (Farrant and Kaila, 2007; Alvarez-Leefmans and Delpire, 2009), and such a differential permeability to HCO_3^- presumably is conferred by GABA_\text{A} receptors with different subunit composition. Future studies will be needed to identify the molecular determinants of HCO_3^- permeability in the GABA_\text{A} receptors of PFs.

The receptors involved in the excitatory presynaptic action of high concentrations of GABA are not blocked by furosemide and therefore are distinct from those that mediate tonic presynaptic inhibition. Although the subunit composition of these receptors also remains to be elucidated, the immunoelectron microscopy data of Stell et al. (2007) indicate that these receptors contain at least the GABA_\text{A} receptor \( \alpha_1 \) subunit.

**Regulation of phasic transmission by tonic inhibition**

In addition to conventional synaptic transmission that relies on presynaptic action potentials to trigger phasic, Ca^{2+}-dependent exocytosis of neurotransmitters, a second mode of communication is based on tonic release of neurotransmitters (most notably GABA) via a mechanism that depends on neither neuronal activity nor Ca^{2+}-dependent exocytosis (Jabaudon et al., 1999; Liu et al., 2000; Rossi et al., 2003) and may be due to channel-mediated release of GABA from glia (Lee et al., 2010). We have discovered that these two mechanisms of synaptic signaling interact, specifically that GABA-mediated tonic inhibition limits phasic glutamate release from PFs. Thus, the low concentrations of ambient GABA present under physiological conditions allows tonic inhibition to fine-tune synaptic circuitry mediated by phasic synaptic transmission.

The logic behind regulation of phasic transmission by tonic inhibition is not yet clear. In general, tonic inhibition is thought to be an adaptive mechanism that alters the input-output gain of a neuron and the excitability of neural networks. Tonic inhibition of phasic synaptic transmission should provide another means of setting the overall tone of a synaptic network. Because the ambient GABA that causes tonic inhibition of PFs appears to be spatially diffuse, this cross talk could provide a spatially diffuse mechanism for controlling neural circuits constructed from phasic synapses. Presynaptic tonic inhibition potentially can also make synaptic information processing sensitive both to changes in ambient GABA signaling as well as to various types of regulators of GABA_\text{A} receptors. Tonic inhibition shows distinct cell specificity and developmental profiles in different brain regions (Semyanov et al., 2004). Tonic inhibition also is regulated by a variety of endogenous and exogenous factors that might provide fine control of phasic synaptic signaling (Stell et al., 2003; Maguire and Mody, 2007) and conceivably could regulate tonic inhibition of presynaptic PFs as well.

In conclusion, we have used the optogenetic indicator, Clo-meleon, to visualize tonic inhibition in the cerebellum. Our finding of tonic inhibition of presynaptic PFs indicates that the ambient GABA level is high enough to activate GABA_\text{A} receptors throughout the cerebellar cortex, including PFs within the ML. This was observed both in slices and in vivo, indicating that this
level of ambient GABA is physiologically relevant. We have found that PF synaptic transmission is under the control of tonic inhibition. Our identification of this novel interaction between phasic and tonic signaling mechanisms suggests that tonic inhibition probably plays a larger than expected role in regulating cerebellar function and perhaps the function of circuits in other brain regions as well.

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