An NMDA Receptor/Nitric Oxide Cascade Is Involved in Cerebellar LTD But Is Not Localized to the Parallel Fiber Terminal

Jung Hoon Shin and David J. Linden
Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Submitted 24 June 2005; accepted in final form 17 August 2005

Shin, Jung Hoon and David J. Linden. An NMDA receptor/nitric oxide cascade is involved in cerebellar LTD but is not localized to the parallel fiber terminal. J Neurophysiol 94: 4281–4289, 2005. First published August 24, 2005; doi:10.1152/jn.00661.2005. Long-term depression (LTD) of the parallel fiber-Purkinje cell synapse in the cerebellum is a cellular model system that has been suggested to underlie certain forms of motor learning. Induction of cerebellar LTD requires a postsynaptic kinase limb involving activation of mGluR1, protein kinase Ca (PKCa), and phosphorylation of ser-880 on the AMPA receptor subunit GluR2. Several lines of evidence have also implicated a complementary phosphatase limb in which N-methyl-D-aspartate (NMDA) receptor-mediated Ca2+ influx activates neuronal nitric oxide synthase (nNOS), the ultimate consequences of which are mediated by nitric oxide (NO), cGMP, and inhibition of postsynaptic protein phosphatases. However, the cellular localization of an NMDA/no cascade has been complicated by the fact that neither functional NMDA receptors nor nNOS are expressed in Purkinje cells. This has lead to a proposal in which NMDA receptors activate nNOS in parallel fibers. Here, we confirm that pharmacological blockade of NMDA receptor or NO signaling blocks induction of LTD. However, no evidence was found for functional NMDA receptors in parallel fiber terminals; blockade of NMDA receptors did not alter either presynaptic Ca2+ transients or the frequency of miniature excitatory postsynaptic currents. NMDA receptor blockade did abolish a slow depolarization evoked by burst stimulation of parallel fiber-stellate cell synapses. The application of NMDA evoked a Ca2+ transient in stellate cell terminals but not in parallel fiber terminals. These results are consistent with the hypothesis that an NMDA receptor/no cascade involved in cerebellar LTD is localized to interneurons rather than parallel fibers.

INTRODUCTION

Excitatory synapses in the brain are often faced with the computational problem of ignoring “background” levels of afferent activity and responding selectively to bursts, which often convey behaviorally salient information. One cellular mechanism to accomplish this involves having receptors that are localized to the perisynaptic region. In this situation, perisynaptic receptors, such as mGluR1 see relatively small glutamate transients from single afferent volleys but are strongly activated by spillover during brief bursts (Batchelor et al. 1994; Brasnjo and Otis 2001; Kim et al. 2003). Another mechanism involves N-methyl-D-aspartate (NMDA) receptors in the postsynaptic density, which require both ligation by glutamate and sufficient postsynaptic depolarization to relieve blockade of the ion channel by Mg2+. These NMDA receptors are poorly activated by single afferent volleys but are effectively activated by either brief afferent bursts or by pairing postsynaptic depolarization with single afferent volleys.

Recently, a new cellular mechanism of burst detection has been proposed at the cerebellar parallel fiber-Purkinje cell synapse, where there are no functional postsynaptic NMDA receptors. It has been proposed that an NMDA receptor/neuronal nitric oxide synthase (nNOS) complex is present in parallel fiber terminals and is activated under conditions where the presynaptic terminal is simultaneously depolarized and exposed to glutamate as might occur during high-frequency bursts (Casado et al. 2000, 2002). In this scheme, glutamate released from the first parallel fiber volley must be bound to parallel fiber NMDA receptors when the second pulse arrives (60 ms later), which will briefly relieve the NMDA receptor Mg2+ block. Ca2+ influx through the NMDA receptor then activates nNOS, and the resultant NO may then diffuse across cell membranes. This is computationally interesting because, unlike a burst detector using postsynaptic NMDA receptors, it constitutes a mechanism that is activated by afferent bursts but not low-frequency pairing of pre- and postsynaptic activity.

In many synapses, burst stimulation can be a trigger for persistent changes in synaptic strength. At the parallel fiber-Purkinje cell synapse, LTD is produced when parallel fiber bursts are paired with activation of the massive, excitatory climbing fiber input (or depolarization of the Purkinje cell to mimic climbing fiber activation). This phenomenon has been suggested to constitute a portion of the engram for forms of motor learning such as adaptation of the vestibuloocular reflex and associative eyelid conditioning (Hansel et al. 2001; Linden 2003).

The molecular requirements for cerebellar LTD induction have begun to be defined. LTD is known to be postsynaptically expressed as it may be detected with exogenous pulses of AMPA receptor agonists (Crepel and Krupa 1988; Ito and Kano 1982; Linden et al. 1991). LTD is not associated with changes in AMPA receptor kinetics, agonist affinity, or unitary conductance (Linden 2001) but is associated with a reduction in AMPA receptor number (Matsuda et al. 2000) by clathrin-mediated endocytosis (Wang and Linden 2000). Triggering of AMPA receptor endocytosis is critically dependent on phosphorylation of a particular residue, ser-880, in the carboxy-terminal PDZ ligand of the AMPA receptor subunit GluR2 (Chung et al. 2003) by PKCa (Leitges et al. 2004).

The phosphorylation state of ser-880 can be controlled by both kinases and phosphatases. The kinase limb involves activation of PKCa by diacylglycerols and Ca2+, the former...
derived from parallel fiber activation of mGluR1/phospholipase C and the latter from climbing fiber activation of voltage-sensitive Ca\(^{2+}\) channels. The phosphatase limb is somewhat less defined but appears to involve a cascade in which nNOS produces NO, which activates soluble guanylyl cyclase. The cGMP produced in this fashion activates cGMP-dependent protein kinase (PKG) that has been proposed to phosphorylate G substrate, thereby increasing its potency as an inhibitor of protein phosphatase 1 (PP1) and PP2A (Ito 2001; Launey et al. 2004). In support of this model, cerebellar LTD in brain slices has been reported to be blocked by manipulations that inhibit NO signaling such as bath application of nNOS inhibitors or NO scavengers or genetic deletion of nNOS (Lev-Ram et al. 1997b; Shibuki and Okada 1991). LTD has also been reported to be blocked by inhibitors of guanylyl cyclase or PKG (Boxall and Garthwaite 1996; Hartell 1994) and mimicked by manipulations that inhibit protein phosphatases (Ajima and Ito 1995; Eto et al. 2002; Launey et al. 2004). In this model, the kinase activation and phosphatase inhibition limbs both work to produce phospho-ser-880 GluR2 and thereby, LTD.

What are the cellular compartments where the NO/cGMP/PKG-signaling cascade occurs? nNOS is a Ca\(^{2+}\)-sensitive enzyme that is often found in a complex with PSD-95 and NMDA receptors (Christopherson et al. 1999). While both PKG and soluble guanylyl cyclase are expressed in Purkinje cells, nNOS is not (Bredt et al. 1990; El-Husseini et al. 1999). nNOS is also absent from cerebellar climbing fibers (Bredt et al. 1990; Vincent and Kimura 1992). This distribution is reflected in experiments with application of drugs to specific compartments: LTD may be blocked by application in the Purkinje cell of inhibitors of PKG and soluble guanylyl cyclase but not nNOS (Hemart et al. 1995; Lev-Ram et al. 1995, 1997a). Extracellular application of NO scavengers that cannot cross cell membranes can block LTD induction (Ito and Karachot 1990; Lev-Ram et al. 1995), implying that NO must diffuse from some other cellular compartment, through the extracellular space, to activate soluble guanylyl cyclase in the Purkinje cell.

Recently, it was shown that bath application of NMDA/glycine gave rise to a large synaptic depression that was blocked by bath application of an nNOS inhibitor and that occluded subsequent LTD induced by parallel fiber depolarization pairing (Casado et al. 2000, 2002). Furthermore, bath application of an NMDA receptor antagonist blocked induction of LTD induced by repeated pairing of Purkinje cell depolarization with a stimulus composed of two parallel fiber volleys delivered with a 60-ms interval. These observations have led to the proposal that parallel fiber bursts during LTD induction activate an NMDA receptor/nNOS-signaling complex in the parallel fiber terminal, giving rise to NO production that could then trigger the phosphatase inhibition limb for LTD in the Purkinje cell (Casado et al. 2002). Here, we have sought to test this hypothesis by combining patch-clamp recording with presynaptic confocal Ca\(^{2+}\) imaging in cerebellar slices.

**METHODS**

**Slice preparation and electrophysiology**

Sagittal (250 \(\mu\)m thick) slices of the cerebellar vermis were prepared from postnatal day 17–19 Sprague-Dawley rats by using a Vibelette 3000 (Vibratome, St. Louis, MO) and standard artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 2.5 KCl, 1.3 MgCl\(_2\), 2.5 CaCl\(_2\), 1 NaH\(_2\)PO\(_4\), 26.2 NaHCO\(_3\), and 20 glucose bubbled with 95% O\(_2\)-5% CO\(_2\) (pH 7.4) at 4°C. Slices were recovered for 30 min in a chamber at 32°C and further recovered for 1 h at room temperature. The slices were placed in a submerged recording chamber that was perfused at a flow rate of 2 ml/min with room temperature ACSF and 5 \(\mu\)M gabazine to block GABA\(_A\) receptors. Visualized whole cell patch-clamp recording was performed with a Zeiss Axioskop FS and a Multiclamp 700A amplifier (Axon Instruments, Union City, CA). The electrodes for Purkinje cell recording (2–4 M\(^{\circ}\)) were filled with a solution containing (in mM) 135 Cs-methanesulfonate, 10 CsCl, 10 HEPEs, 0.2 EGTA, 4 Na\(_2\)-ATP, and 0.4 Na-GTP (pH 7.2). For stable recordings of Purkinje cell miniature excitatory postsynaptic currents (mEPSCs), we used a different solution containing 88 Cs\(_2\)SO\(_4\), 10 EGTA, 4 MgSO\(_4\), 4 CaCl\(_2\), 1.5 MgCl\(_2\), 4 Na\(_2\)-ATP, 0.3 Na\(_2\)-GTP, and 0.1 D600 (pH 7.2) (Dittman and Regehr 1996). Cells were voltage-clamped at −70 mV unless otherwise indicated. The currents were filtered at 2 kHz and digitized at 10 kHz. For current-clamp recording from stellate cells, electrodes (6–8 M\(^{\circ}\)) were filled with a solution containing (in mM) 130 K-methanesulfonate, 10 NaCl, 2 MgCl\(_2\), 10 HEPEs, 0.2 EGTA, 4 Na\(_2\)-ATP, and 0.4 Na-GTP (pH 7.2). For extracellular stimulation, standard patch pipettes filled with ACSF were used. Parallel fibers were stimulated in the molecular layer. Test stimulation was given using paired-pulses (100-ms interval) at a frequency of 0.1 Hz using 80–120 \(\mu\)A pulses (100-\(\mu\)s duration). Stimulus strength was adjusted so that the first EPSC did not exceed 300 pA for Purkinje cells. For stellate cell recordings, parallel fiber test pulses with similar intensities were used. To induce LTD, parallel fibers were stimulated with a train of five pulses at 100 Hz, which was accompanied by 100-ms-long depolarization of the Purkinje cell to 0 mV (see Fig. 1C). A total of 30 trains were made at 2-s intervals. For mEPSC analysis, a template was made to detect events in pClamp9 software (Axon Instruments), by averaging ~30 hand-picked mini events. When detecting events, the template match threshold was set to 4. SR 95531 hydrobromide (gabazine), D-2-amino-5-phosphonopentanoic acid (D-AP5), N\(^{\\text{N}}\)-nitro-l-arginine (L-NNA), 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxide (c-PTIO), MK-801, baclofen, NMDA, and NBQX (disodium salt) were purchased from Tocris Cookson (Ellisville, MO), and TTX from Calbiochem (San Diego, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

**Parallel fiber presynaptic calcium imaging**

The method of loading the Ca\(^{2+}\) indicator into parallel fibers was adapted from Regehr and Atluri (1995). Briefly, 50 \(\mu\)g of either Fluo-4/AM or Oregon Green BAPTA-1/AM (OGB-1) from Molecular Probes (Eugene, OR) was dissolved in 20 \(\mu\)l of 20% Pluronic in DMSO and was then added to 400 \(\mu\)l of filtered ACSF. The mixture was vortexed and sonicated for 1 min each. The Fluo-4 solution was used to fill a glass pipette with a 6–8 \(\mu\)m tip and was ejected into the molecular layer of a transverse slice (300 \(\mu\)m) by constant positive pressure for 30 min. The direction of ejection was at a right angle to the course of the parallel fibers. A suction pipette with a ~15-\(\mu\)m tip was placed near to the ejection site to remove excess dye. Ca\(^{2+}\) transients at areas ~400 \(\mu\)m from the delivery site were recorded by a laser scanning confocal microscope (Zeiss LSM 510) with a \(\times40\) water-immersion objective lens. Fluo-4 was excited with the 488-nm line of an Argon ion laser. Emitted fluorescence was collected through a 505-nm long-pass filter. Fluo-4 images were recorded in line-scan mode with 512 pixels per line at 500 Hz for 2 s. The scan line was oriented at a right angle to the course of the parallel fibers. Parallel fibers were stimulated with either paired-pulses (60 ms apart) or a train of five pulses at 100 Hz that began 480 ms after the onset of line-scanning. Ca\(^{2+}\) transients are presented as \(\Delta F/F_0\). Line-scanned
images were analyzed off-line with Igor software (WaveMetrics, Lake Oswego, OR) and Origin software (OriginLab, Northhampton, MA).

Stellate cell presynaptic calcium imaging

For stellate cell Ca\(^{2+}\)/H11001 imaging, 0.2 mM EGTA in the pipette solution was replaced by 0.4 mM Fluo-4, and 1 mM Alexa 594 hydrazide (Molecular Probes, Eugene, OR) was added to visualize axon terminals. To minimize phototoxicity, 0.1 mM Trolox-C was added to ACSF, and the power and exposure time of laser illumination were maintained as low as possible. For the experiments in which NMDA/glycine was applied, a cocktail of blockers were added (in μM): 1 CGP55845, 0.5 DPCPX, and 1 strychnine. Ca\(^{2+}\)/H11001 transients were elicited by a burst of five brief current injections (2-ms long with 20-ms interval) every 2 min and recorded by a laser scanning confocal microscope (Zeiss Pascal) with a \(40\) water-immersion objective lens. Fluo-4 was excited with the 488 nm line of an Argon ion laser, and emitted fluorescence was collected through a 505-nm long-pass filter. Alexa 594 hydrazide was excited with the 543-nm line of a He-Ne laser, and the fluorescence was collected through a 560 long-pass filter. Fluo-4 images were recorded in frame-scan mode with 128 \(\times\) 52 pixels at 20 Hz. For analysis, foreground pixels were determined by thresholding the image, and spatially averaged to calculate \(\Delta F/F_0\) for each frame.

RESULTS

Using the whole cell voltage-clamp technique, we recorded currents from the somata of Purkinje cells from 250-μm-thick sagittal slices of P17-19 rats. Purkinje cells were held at \(-70\) mV. A stimulation electrode was placed in molecular layer, and a paired-pulse test stimulus (100-ms interval) every 10 s and evoked excitatory postsynaptic currents (EPSCs) were recorded from Purkinje cells in sagittal slices. A, middle: amplitude of the 1st EPSC (filled circles) and the paired-pulse ratio (PPR, empty circles) were normalized and plotted as a function of time. Each point represents the average of 10 consecutive episodes. Black box at \(t = 0\) min, pairing of a parallel fiber burst with Purkinje cell depolarization, which is further illustrated with a schematic diagram as shown in C. Top: superimposed traces are the averages of 10 consecutive episodes recorded immediately before (black) and 5 min (blue) or 25 min after (red) LTD induction, as denoted by asterisks. Bottom: EPSC amplitudes and PPRs from individual experiments were normalized and pooled (mean \(\pm\) SE, \(n = 5\)). B, \(\alpha\)-AP5 (50 μM) was bath-applied at least for 10 min before recording, \(n = 7\) cells (bottom).

FIG. 1. An N-methyl-d-aspartate (NMDA) receptor antagonist, \(\alpha\)-2-amino-5-phosphonopentanoic acid (\(\alpha\)-AP5), blocks the induction of cerebellar long-term depression (LTD). Parallel fibers were stimulated with a paired-pulse (100-ms interval) every 10 s and evoked excitatory postsynaptic currents (EPSCs) were recorded from Purkinje cells in sagittal slices. A, middle: amplitude of the 1st EPSC (filled circles) and the paired-pulse ratio (PPR, empty circles) were normalized and plotted as a function of time. Each point represents the average of 10 consecutive episodes. Black box at \(t = 0\) min, pairing of a parallel fiber burst with Purkinje cell depolarization, which is further illustrated with a schematic diagram as shown in C. Top: superimposed traces are the averages of 10 consecutive episodes recorded immediately before (black) and 5 min (blue) or 25 min after (red) LTD induction, as denoted by asterisks. Bottom: EPSC amplitudes and PPRs from individual experiments were normalized and pooled (mean \(\pm\) SE, \(n = 5\)). B, \(\alpha\)-AP5 (50 μM) was bath-applied at least for 10 min before recording, \(n = 7\) cells (bottom).
maintain the integrity of the Purkinje cell dendrites and a Cs-based internal saline to improve the voltage clamp. As shown in Fig. 1B, bath application of 50 μM D-AP5 blocked the induction of LTD. In fact, the EPSC was transiently potentiated (136.9 ± 4.6% at t = 5 min, n = 8) after the pairing stimulation and returned to baseline later (107.8 ± 8.7% at t = 28 min). The potentiation of EPSCs after the failed LTD induction in the presence of D-AP5 may be partly due to a change in the probability of release as it was mirrored by a small, transient decrease in PPR. We also found that LTD induction was blocked by another NMDA receptor antagonist, MK-801 (50 μM; 94.3 ± 8.5% at t = 28 min; n = 6).

One possible downstream effector of NMDA receptors is nNOS activation and the consequent production of NO. We have tested the involvement of NO in LTD by using an nNOS inhibitor and a nitric oxide (NO) scavenger (carboxy-PTIO, 30 μM). When parallel fiber/depolarization pairing was given after bath-application of either of these compounds, LTD was blocked (Fig. 2). After parallel fiber/depolarization pairing, the EPSCs were transiently potentiated (118.8 ± 13.9%, n = 3, and 162.7 ± 10.9%, n = 4, at t = 5 min) and returned to the baseline in L-NNA (93.5 ± 15.7% at t = 28 min) or remained potentiated in c-PTIO (135.0 ± 11.8%). A transient decrease in PPR was observed in both conditions. Thus we have replicated previous work implicating NMDA receptor activation (Casado et al. 2002) and NO production (Casado et al. 2002; Ito and Karachot 1990; Shibuki and Okada 1991) in cerebellar LTD.

To address the proposal that functional NMDA receptors are present in parallel fiber terminals and are activated by bursts or pulse pairs (Casado et al. 2000, 2002), we sought to measure parallel fiber Ca2+ transients (Regehr and Atluri 1995). After loading the Ca2+-sensitive dye Fluo-4, into parallel fibers in a transverse slice, Ca2+ transients were recorded by a laser scanning confocal microscope along a line oriented at right angles to the parallel fibers. Figure 3(A, top) is a representative line-scan image where the x axis is time and the y axis is the line-scan coordinate. The spatial average of the fluorescence intensity along the y axis is the normalized average amplitude from populations (middle) and PPRs (bottom) from representative cells (middle), and normalized average amplitude from populations (bottom, n = 4 cells/group).
It has been reported that d-AP5 reversibly reduced the frequency of mEPSCs recorded from pyramidal neurons of the visual (Sjostrom et al. 2003) or entorhinal (Berretta and Jones 1996) cortex. These findings suggest that Ca\textsuperscript{2+} influx through presynaptic NMDA receptors can elevate mEPSC frequency by contributing to resting Ca\textsuperscript{2+} levels in the presynaptic terminal. To assess whether this action of presynaptic NMDA receptors exists in parallel fiber-Purkinje cell synapses, mEPSCs were recorded from Purkinje cells in the presence of 500 nM tetrodotoxin (TTX). When d-AP5 (50 μM) was applied, the frequency of mEPSCs did not significantly change (106.5 ± 5.1% baseline at t = 14 min, n = 6, Fig. 4A). However, when the GABA\textsubscript{B} receptor agonist baclofen (5 μM) was applied as a positive control, mEPSC frequency decreased (77.6 ± 8.4% baseline at t = 24 min). We then tried more permissive conditions in an attempt to reveal an effect of NMDA receptor antagonists on mEPSC frequency: the external concentration of Mg\textsuperscript{2+} was reduced to 0.1 mM. However, even with the potential Mg\textsuperscript{2+} block of NMDA receptors greatly reduced, neither d-AP5 nor (R)-CPP (20 μM) produced significant changes in mEPSC frequency (96.5 ± 6.2% (n = 6) and 108.9 ± 8.8% (n = 6) of baseline at t = 15 min, respectively, Fig. 4B). In these experiments, 5 μM CGP55845, GABA\textsubscript{B}-receptor antagonist, was added to eliminate potential indirect effects from GABAergic interneurons.

The most permissive screen for a linkage between parallel fiber NMDA receptors and glutamate release is to bath apply NMDA in the presence of low external Mg\textsuperscript{2+} while measuring Purkinje cell mEPSCs. This would be expected to be both directly and persistently open NMDA receptor-associated ion channels. In this experiment, NMDA (30 μM) failed to alter the frequency of mEPSCs (98.3 ± 6.1% baseline at t = 15 min, n = 5, Fig. 5, A and B). This treatment also failed to evoke an increase in basal parallel fiber Ca\textsuperscript{2+} concentration (96.8 ± 3.3% of baseline, n = 6, Fig. 5C). Taken together, these negative results with NMDA receptor agonists and antagonists using both mEPSCs and parallel fiber Ca\textsuperscript{2+} imaging, argue against the hypothesis that functional NMDA receptors are present in the parallel fiber terminal.

If the NMDA receptors that contribute to cerebellar LTD do not reside in either the parallel fiber terminal or the Purkinje cell dendrite, where might they be located? One possible location for functional NMDA receptors is in the somatodendritic compartment of stellate cells, which also receive synaptic inputs from parallel fiber terminals. To investigate this hypothesis, we recorded excitatory postsynaptic potentials (EPSPs) evoked by parallel fiber burst stimulation in stellate cells (bursts consisted of 5 stimuli at 100 Hz delivered in 1.33 min, Fig. 6). When 20 μM NBQX was applied, fast AMPA receptor-mediated responses were abolished, but a slow EPSP was persistent. This slow component was reversibly blocked by d-AP5, suggesting that stellate cell NMDA receptors become activated by the bursts used for LTD induction. This is consistent with previous reports that burst stimulation recruits extrasynaptic NMDA receptors on cerebellar interneurons (Carter and Regehr 2000; Clark and Cull-Candy 2002).

Previous work has shown that bath application of NMDA can increase mIPSC frequency recorded in Purkinje cells (Duguid and Smart 2004; Glitsch and Marty 1999; Huang and Bordey 2004), presumably through activation of interneuronal NMDA receptors. As these recordings were made in TTX, it is presumed that this effect mostly results from activation of NMDA receptors in the interneuron terminals. However, it has been suggested that a portion of this effect on miniature inhibitory postsynaptic current (mIPSC) frequency results from activation of NMDA receptors in the somato-dendritic region of interneurons as well (Glitsch and Marty 1999).

One report (Huang and Bordey 2004) found a reversible decrease in mIPSC amplitude with d-AP5 (and 1 mM external Mg\textsuperscript{2+}), suggesting that, at least in their conditions, there was a tonic level of NMDA receptor activation in interneurons. To address this possibility, we measured internal Ca\textsuperscript{2+} from the...
presynaptic terminals of stellate cells loaded with Fluo-4 and bathed in the GABA<sub>B</sub> receptor antagonist CGP 55845 (5 μM). Under current-clamp mode, the cells were injected with a train of five current steps (2-ms long with an interval of 20 ms) to elicit a reproducible burst of five action potentials (Fig. 6B, left). As shown in Fig. 6B (middle), this evoked a substantial Ca<sup>2+</sup> transient that slowly returned to the baseline. After 10 min of recording, 50 μM D-AP5 was applied, and this treatment affected neither the evoked calcium transients (107.4 ± 10.1% baseline at t = 20 min, n = 4, Fig. 6B, right) nor the basal Ca<sup>2+</sup> concentration (94.7 ± 6.0% baseline at t = 20 min). These results suggest that, in our hands, tonic NMDA receptor activation on interneuron presynaptic terminals was minimal. However, the presence of interneuron terminal NMDA receptors was confirmed by external application of NMDA (30 μM) in the presence of 1.3 mM Mg<sup>2+</sup> which evoked a small but significant increase in basal Ca<sup>2+</sup> in stellate cell terminals (117.8 ± 7.8% baseline, n = 7; P < 0.05 by t-test, Fig. 6C). Thus functional NMDA receptors appear to be present in both somato-dendritic and axonal compartments of stellate cells.

DISCUSSION

The main finding of these investigations is that a functional NMDA receptor/nNOS complex is not present in parallel fiber terminals; neither blockade nor activation of NMDA receptors altered parallel fiber Ca<sup>2+</sup> transients nor the frequency of mEPSCs recorded in Purkinje cells. Therefore the contribution of this signaling unit to burst detection and the induction of cerebellar LTD must occur in a different cellular compartment. The observation that parallel fiber bursts of the kind used during LTD induction activate NMDA receptors in stellate interneurons is consistent with the hypothesis that these cells (and perhaps basket cells as well) are the source of NMDA receptor-triggered NO production that is required for induction of cerebellar LTD.

These results are also consistent with some previous immunocytochemical and electrophysiological findings. NMDA receptor subunit immunoreactivity is expressed weakly and only in a small subset of parallel fibers (Petralia et al. 1994) but strongly in interneurons (Akazawa et al. 1994). nNOS immunoreactivity is weak or absent in parallel fibers but is strong in both stellate and basket cells (in both somata and terminals) (Rodrigo et al. 2001). Furthermore, bath application of NMDA did not produce an alteration in the probability of glutamate release as indexed by the PPR of parallel fiber-Purkinje cell EPSCs (Casado et al. 2000).

Is it possible to reconcile the present results with the parallel fiber localization of an NMDA receptor/nNOS cascade by assuming compartmentalization of the parallel fiber terminals? For this to hold, Ca<sup>2+</sup> influx through NMDA receptors would...
have to be able to activate nNOS but be invisible to both the release machinery (as indexed by both mEPSC frequency and paired-pulse facilitation) and confocal Ca2+ imaging (even when evoked by a burst of five pulses at 100 Hz in Mg2+-free saline or bath NMDA application). While this is formally possible, we believe that it is unlikely and would require an unprecedented degree of cytosolic compartmentalization beyond what we (Fig. 6) and others (Duguid and Smart 2004; Glitsch and Marty 1999; Huang and Bordey 2004) have observed for NMDA receptors in the terminals of cerebellar interneurons. Furthermore, other manipulations which attenuate Ca2+ channels in parallel fiber terminals, produce reductions in parallel fiber-evoked presynaptic Ca2+ transients. These include agonists of adenosine A1 receptors (Dittman and Regehr 1996), GABA_B receptors (Dittman and Regehr 1996), and CB1 cannabinoid receptors (Kreitzer and Regehr 2001).

Parallel fiber stimulation does not appear to activate presynaptic parallel fiber NMDA receptors but does activate NMDA receptors on stellate cells. However, it is unclear whether an interneuronal NMDA receptor/nNOS complex would function in the somato-dendritic membrane of the interneuron as driven by synapses from parallel fibers or in the presynaptic terminals of the interneuron-Purkinje cell synapse or both. In support of the latter possibility, several laboratories have found that bath application of NMDA produced an increase in the frequency of mIPSCs recorded in Purkinje cells that could be blocked by an NMDA receptor antagonist (Duguid and Smart 2004; Glitsch and Marty 1999; Huang and Bordey 2004). nNOS (Rodrigo et al. 2001) and PSD-95 (McGee et al. 2001) immunoreactivity have also been reported in interneuron presynaptic terminals and could therefore potentially form a signaling complex with NMDA receptors at this location. The recordings illustrated in Fig. 6 confirm the presence of functional NMDA receptors in both somato-dendritic and axonal compartments of stellate cells but do not allow us to determine which of these receptors are important in induction of parallel fiber-Purkinje cell LTD.

To this point, we have assumed that the source of glutamate for activating interneuron NMDA receptors during LTD induction is the parallel fiber. However, it should be mentioned that there are lines of evidence suggesting that depolarization-evoked release of glutamate from Purkinje cell dendrites may function as a retrograde signal to activate interneuron NMDA receptors. For example, Duguid and Smart (2004) report that the interneuronal presynaptic NMDA receptors are involved in a transient enhancement of GABA release triggered by Purkinje cell depolarization and a subsequent postsynaptic Ca2+ transient.

At present, the argument that interneuronal NMDA receptors drive the NO signal that contributes to cerebellar LTD is one of exclusion. We have no mechanism at hand for interfering with the NO cascade specifically in stellate and/or basket cells that would provide a definitive test of this hypothesis. Optical measurement of NO levels in cerebellar slices would be useful to address the question of where NO is produced, but in pilot experiments, we found that the NO dyes currently available have technical issues that preclude their use in these experiments.

A consequence of a model for LTD induction with complementary kinase activation and phosphatase inhibition limbs (Fig. 7) is that in conditions of low basal phosphatase activity, kinase activation may be sufficient to produce LTD, whereas in high basal phosphatase activity, both kinase activation and phosphatase inhibition may be required for LTD. The former may be the case in cultured Purkinje cells, where inhibition of NO/cGMP/PKG signaling fails to block LTD (Linden et al. 1995), possibly due to reduced activation of interneurons. In cultured Purkinje cells, PKC itself has the ability to inhibit the myosin/moesin phosphatase form of PP1 through phosphorylation of the inducible inhibitor CPI-17 (Eto et al. 2002). It will
be interesting to determine whether the PKC/CPI-17 mechanism is also operative in cerebellar slices.

The present model suggests that both the kinase activation limb and the phosphatase inhibition limb for cerebellar LTD induction have the ability to ignore low-frequency activity but detect bursts. Interestingly, this appears to occur by different mechanisms (mGluR1 activation for the kinases vs. NMDA receptor activation for phosphatases) and in different cellular compartments (Purkinje cell dendrites vs. interneurons). Placing the NMDA/nNOS module in interneurons allows for a unique “feed-forward” circuit for LTD induction at the parallel fiber-Purkinje cell synapse. In addition, it may also provide a signal for another form of plasticity. Recently, it has been shown that an NO-dependent form of LTP may be produced at parallel fiber-stellate cell synapses by pairing postsynaptic depolarization with 2-Hz stimulation of parallel fibers (Rancil- lac and Crepel 2004). Thus activation of an NMDA receptor/nNOS module in cerebellar interneurons may ultimately serve to persistently attenuate Purkinje cell activity in two different ways: through LTD of parallel fiber-Purkinje cell synapses and by LTP of parallel fiber-interneuron synapses, the latter of which will result in increased Purkinje cell inhibition by interneurons.

**FIG. 6.** Functional NMDA receptors in the somato-dendritic and presynaptic terminal regions of cerebellar stellate interneurons. A: parallel fibers were stimulated with a burst (a train of 5 at 100 Hz). The somatic voltage was recorded from stellate cells under current clamp in the presence of the AMPA/kainate receptor antagonist (NBQX, 20 μM). D-AP5 (50 μM) was then washed in and out. B, left: a train of current injections (2-ms-long pulses with and interpulse interval of 20 ms, repeated 5 times) was delivered to the stellate cell every 2 min. This test stimulus generated a reproducible burst of action potentials. Two traces (black: pretreatment and red: +50 μM D-AP5) are superimposed to show that there are no significant changes in the action potential waveform after D-AP5. Middle: when the current injection was delivered, the presynaptic Ca2+ transients in stellate cell terminals loaded with Fluo-4 were measured simultaneously with a confocal microscope operating in frame scanning mode at 20 Hz. The 2 images shown below the traces are false color “snap shots” obtained before (bottom) and right after (top) the current injection. ΔF/F₀ was calculated from the foreground pixels within the ROI indicated by a yellow box (see METHODS). Right: areas under the Ca2+ transient were calculated and pooled to produce a normalized mean ± SE (n = 4).

**FIG. 7.** A simplified model of LTD induction at the parallel fiber-Purkinje cell synapse. A kinase activation limb involving mGluR1 and protein kinase C (PKC) is located in the Purkinje cell dendritic spine. A phosphatase inhibition limb is initiated by activation of interneuron NMDA receptors and the consequent activation of interneuronal nNOS. NO diffuses from interneurons to the Purkinje cell dendritic spines to activate soluble guanylyl cyclase and ultimately produce phosphatase inhibition.
ACKNOWLEDGMENTS

R. Bock provided excellent technical support. Useful critique was provided by D. Bergles, H. Nishiyama, Y. Shen, W. Zhang, A. Sdrulla, S. J. Kim, and S. Gardiner.

GRANTS

This work was supported by National Institute of Mental Health Grant R37 MH-51106 and the Develbiss Fund.

REFERENCES


