Presynaptic Current Changes at the Mossy Fiber–Granule Cell Synapse of Cerebellum During LTP

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Maffei, Arianna, Francesca Prestori, Paola Rossi, Vanni Taglietti, and Egdio D’Angelo. Presynaptic current changes at the mossy fiber–granule cell synapse of cerebellum during LTP. J Neurophysiol 88: 627–638, 2002; 10.1152/jn.00879.2001. The involvement of presynaptic mechanisms in the expression of long-term potentiation (LTP), an enhancement of synaptic transmission suggested to take part in learning and memory in the mammalian brain, has not been fully clarified. Although evidence for enhanced vesicle cycling has been reported, it is unknown whether presynaptic terminal excitability could change as has been observed in invertebrate synapses. To address this question, we performed extracellular focal recordings in cerebellar slices. The extracellular current consisted of a pre- (P1/N1) and postsynaptic (N2/SN) component. In ~50% of cases, N1 could be subdivided into N1a and N1b. Whereas N1a was part of the fiber volley (P1/N1a), N1b corresponded to a Ca2+-dependent component accounting for 40–50% of N1, which could be isolated from individual mossy fiber terminals visualized with fast tetramethylindocarbocyanine perchlorate (DiI). The postsynaptic response, given its timing and sensitivity to glutamate receptor antagonists [N2 was blocked by 10 μM [1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzof][quinoxaline-7-sulfonamide disodium (NBQX) and SN by 100 μM APV + 50 μM 7-CI-kyn], corresponded to granule cell excitation. N2 and SN could be reduced by 1) Ca2+ channel blockers, 2) decreasing the Ca2+ to Mg2+ ratio, 3) paired-pulse stimulation, and 4) adenosine receptor activation. However, only the first two manipulations, which modify Ca2+ influx, were associated with N1 (or N1a) reduction. LTP was induced by θ-burst mossy fiber stimulation (8 trains of 10 impulses at 100 Hz separated by 150-ms pauses). Interestingly, during LTP, both N1 (or N1a) and N2/SN persistently increased, whereas P1 (or P1/N1a) did not change. Average changes were N1 = 38.1 ± 31.9, N2 = 49.6 ± 48.8, and SN = 59.1 ± 35.5%. The presynaptic changes were not observed when LTP was prevented by synaptic inhibition, by N-methyl-D-aspartate and metabotropic glutamate receptor blockade, or by protein kinase C blockage. Moreover, the presynaptic changes were sensitive to Ca2+ channel blockers (1 mM Ni2+ and 5 μM ω-CTx-MVIIC) and occluded by K+ channel blockers (1 mM tetraethylammonium). Thus regulation of presynaptic terminal excitability may take part in LTP expression at a central mammalian synapse.

INTRODUCTION

There is open debate about the expression mechanism of long-term potentiation (LTP), an enhancement of synaptic transmission suggested to take part in learning and memory in the mammalian brain (Bliss and Collingridge 1993; Hawkins et al. 1993; Malenka and Nicoll 1999). A major question is whether LTP depends on pre- or postsynaptic changes. The involvement of presynaptic expression mechanisms during LTP has been indirectly supported by quantal analysis of postsynaptic responses (Bekkers and Stevens 1990; Malagolri and Tsiens 1992) and by the involvement of retrograde messengers backpropagating from the postsynaptic induction site (Gretwaite et al. 1988; Schuman and Madison 1991). Recently, enhanced presynaptic protein expression and uptake of fluorescent dyes have been proposed as evidence for increased vesicle cycling during LTP (Malagolri et al. 1995; Naya et al. 1996; Zakharineko et al. 2001). Enhanced neurotransmitter release could also involve changes in presynaptic terminal currents; e.g., an increase in Ca2+ or a decrease in K+ currents as observed in some forms of plasticity in Aplysia and other invertebrates (Hawkins et al. 1993; Kandel and Schwartz 1982). Although Ca2+ and K+ currents control neurotransmitter release at central mammalian synapses also (e.g., Ishikawa and Takahashi 2001; Lærum and Storm 1994), no changes in presynaptic terminal excitability have been reported so far during LTP.

A prerequisite for this investigation is that stable long-lasting recordings are established from presynaptic terminals and that a simultaneous monitoring of the afferent volley and postsynaptic response is obtained. To this aim we have performed extracellular focal recordings (Del Castillo and Katz 1956), which have previously been used to measure presynaptic currents at the neuromuscular junction (Angaut-Petit et al. 1989; Brigant and Mallart 1982; Del Castillo and Katz 1956; Katz and Miledi 1965; Mallart 1985) and spine currents in cultured neurons (Forti et al. 1997). With focal recordings, we have investigated neurotransmission and LTP at the cerebellar mossy fiber–granule cell synapse. Cerebellar mossy fibers form large glutamatergic terminals that contact numerous granule cells (D’Angelo et al. 1995; Eccles et al. 1967; Garthwaite and Brodbelt 1989; Palay and Chan-Palay 1974), and their high-frequency stimulation causes N-methyl-D-aspartate (NMDA) receptor-dependent LTP (Armano et al. 2000; D’Angelo et al. 1999; Hansel et al. 2001). Similar to a few other examples in the mammalian brain (Borst and Sackmann 1998; Geiger and
Jonas 2000), the large size of cerebellar mossy fibers makes them suitable candidates for measuring extracellular currents.

In the present study, we show that during LTP, the current generated by cerebellar mossy fiber terminals was persistently increased. The presynaptic change depended on the same mechanisms that determined LTP induction in granule cells, including activation of the NMDA receptor, metabotropic glutamate receptor (GluR)-1, and protein kinase C (PKC) (D’Angelo et al. 1999; Masgrau et al. 2001; Rossi et al. 1996). The presynaptic change was correlated with the intensity of LTP, was sensitive to Ca\(^{2+}\) channel blockers, and was occluded by K\(^{+}\) channel blockers. Moreover, although it occurred after manipulations of presynaptic Ca\(^{2+}\) influx, no presynaptic change followed direct manipulation of neurotransmitter release through paired-pulse stimulation (PPS) or adenosine receptor activation. These results provide direct evidence that presynaptic excitability changes, which may enhance neurotransmission during LTP, take place at cerebellar glomerular synapses.

**METHODS**

Focal current recordings (Brigart and Mallart 1982; Del Castillo and Katz 1956; Forti et al. 1997; Katz and Miledi 1965; Mallart 1985) were performed in acute 250-μm-thick cerebellar slices obtained from 19- to 22-day-old Wistar rats, as reported previously (Armano et al. 2000; D’Angelo et al. 1995, 1999). Slices were maintained in standard Krebs solution at 30°C. Unless otherwise stated, the solutions contained the GABA\(_A\) receptor blocker bicuculline (10 μM; Sigma, St. Louis, MO). Drugs were either perfused in the bath [6-cyano-7-nitroquinoxalene-2,3-dione (CNQX), 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzof]quinoline-7-sulfonamide disodium (NBQX), 2-amino-5-phosphonovaleric acid (APV), 7-chlorokynurenine acid, (RS)-1-amino-IND-1,5-dicarboxylic acid (AIDA; Tocris Cookson), C6-adenosine, TTX (Sigma)] or through a local pipette [o-CTX-MVIIIC (Bachem, Heidelberg, Germany) and GO 6983 (Calbiochem)] with a 50- to 100-μM tip diameter. The mossy fiber bundle was stimulated with a bipolar tungsten electrode via a stimulus isolation unit (0.2-ms voltage pulses at 0.1 Hz), and a patch-pipette (5–10 μM) was gently positioned on the slice until an active spot was detected (Del Castillo and Katz 1956; Katz and Miledi 1965). Once the recording pipette contacted the slice, a ‘gap resistance’ nearly doubled the circuit load. This gap resistance was monitored by measuring current deflections generated by small voltage steps throughout the recordings (recordings with >25% changes in gap resistance were rejected). Inward membrane currents collected from below the electrode appeared as upward deflections and were termed N (negative) conforming to the nomenclature of cerebellar field recordings (Eccles et al. 1967; Garthwaite and Brodbelt 1989). Patch-clamp technology was adopted to ensure a wide signal-to-noise ratio and recording bandwidth. Focal extracellular currents were recorded with an Axopatch 200B amplifier in the voltage-clamp mode (0 mV command potential) at a 10-kHz cutoff frequency (–3 dB) and were sampled with a Digidata 1200B interface at 50 μs/point (Axon Instruments, Union City, CA). Patch-pipettes ensured low tip resistance (1–2 MΩ with Krebs filling solution), and fire-polishing was used to improve the gap resistance, increasing the current measured by the amplifier. Capacitive coupling and stimulus artifact were reduced by using thick-walled borosilicate (hard-glass) capillaries (Hingelberg, Malsfeld, Germany) and by Sylgard-coating the pipettes close to their tips. The extracellular signals were monitored on-line with LTP101M software (kindly provided by Dr. William Anderson; University of Bristol, UK), converted to an appropriate format and analyzed off-line with pClamp software. Signal quality was improved by averaging 10 consecutive tracings, and the stimulus artifact (which was isolated by applying 1 μM TTX at the end of the recordings) was subtracted. Peak amplitude was taken as a measure of N\(_1\) and N\(_2\). Except for exemplar recordings shown in Figs. 1–3, amplitude calibration was omitted because signal amplitude depended on factors unrelated to membrane currents. Data in the text are reported as means ± SD.

Mossy fibers and their terminals were identified under fluorescence optics (CCD camera; PCCO Sensicam, Martinsried, Germany and monochromator; TILL Photonics, Leiden, The Netherlands) on the mossy fiber bundle. After locating a presynaptic terminal and its axon, the microscope was switched to Nomarsky optics, and a recording (2- to 4-μM diameter) and a stimulating (1-μM diameter) pipette were positioned with digitally controlled piezoelectric manipulators (Physik Instrumente, Waldbronn, Germany), allowing recordings to be made from individual mossy fiber terminals.

LTP was induced by θ-burst stimulation (TBS; 8 bursts of 10 impulses at 100 Hz repeated every 250 ms) after 20 min of control stimulation at 0.1 Hz (Armano et al. 2000; D’Angelo et al. 1999).

**RESULTS**

Focal current recordings from the cerebellum granular layer

Focal current recordings (Brigart and Mallart 1982; Del Castillo and Katz 1956; Forti et al. 1997; Katz and Miledi 1965; Mallart 1985) were obtained by positioning the recording electrode in the granular layer while stimulating the mossy fiber bundle within the folium [intrafolial stimulation (IFS); Figs. 1A and 2A]. The focal current typically comprised four components termed P\(_1\), N\(_1\), N\(_2\), and SN, resembling those recorded with glove-gate recordings in vitro (Garthwaite and Brodbelt 1989) and field recordings in vivo (Eccles et al. 1967) (Figs. 1A and 2A). In 46% of cases, N\(_1\) showed a single peak at 1.2 ± 0.4 ms (n = 34; for example, see Fig. 1C), whereas in the remaining cases, N\(_1\) displayed a main peak at 0.7 ± 0.3 ms (N\(_1\mu\)) and a secondary peak at 1.5 ± 0.2 ms (N\(_{1b}\); n = 40; for examples, see Figs. 1A, 2B, and 3D). N\(_2\) arose at 2.1 ± 0.3 ms (n = 74) and peaked at 3.3 ± 1.1 ms (n = 74). N\(_2\)/SN apparently changed from an excitatory postsynaptic potential (EPSP) to an EPSP–spike complex as the stimulus intensity was increased (Fig. 1A). A comparison with voltage tracings recorded in patch clamp, whole cell recordings showed that N\(_2\)/SN was in phase with EPSP–spike complexes measured postsynaptically from granule cells (Armano et al. 2000; D’Angelo et al. 1995, 1999) (Fig. 1B), whereas P\(_1\)/N\(_1\) fell within the synaptic delay.

To determine whether the focal current (especially N\(_2\)) received a contribution from Purkinje cells (Eccles et al. 1967), in some experiments, either the Purkinje cell layer was removed (IF\(S_{cal}\)) or mossy fibers were stimulated through their collateral branches in neighboring folia [transfolial stimulation (TFS)], thus preventing Purkinje cell axon activation (Fig. 1C). In these experiments, the focal current was similar to that obtained by IFS, as reflected by the similarity of the corresponding N\(_2\)/N\(_1\) ratios [IFS\(_{cal}\) 1.28 ± 1.2 (n = 5), TFS 1.45 ± 0.6 (n = 4), IFS 1.43 ± 0.8 (n = 13)]. Another way to unveil the contribution of Purkinje cells is to cause their inhibition through molecular layer interneurons (Eccles et al. 1967). This was done in experiments wherein bicuculline was omitted from the extracellular solution and parallel fibers were activated by a second stimulating electrode molecular layer stimulation (MLS). Although molecular layer conditioning caused a strong granule cell inhibition through Golgi cells (see following text and Fig. 1D), no changes were observed in N\(_1\).

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Golgi cells may contribute to shape the focal current, because both their axons and dendrites enter the glomeruli, making connections with granule cells and mossy fibers, respectively (Eccles et al. 1967). In fact, in the absence of bicuculline, mossy fiber stimulation elicited a large inhibitory response 3–5 ms after the stimulus (Fig. 1D), which decayed with a time course reflecting granule cell inhibition. However, \( N_1 \) was unaffected. These results indicate that the focal current elicited by IFS mostly reflected mossy fiber and granule cell excitation.

Unless differently stated, IFS will be adopted in the following experiments.

**Pharmacological and functional properties of the focal current**

The nature of pre- and postsynaptic waveforms was clarified by pharmacological experiments. Because granular layer excitation is glutamatergic (Garthwaite and Brodbelt 1988), the
FIG. 2. Pharmacological and functional properties of focal currents. A: application of N-methyl-D-aspartate (NMDA) receptor antagonists APV (100 μM) and 7-Cl-kyn (50 μM) reduced SN more markedly than N2. Addition of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo[1]quinoxaline-7-sulfonamide disodium (NBQX; 10 μM) fully blocked the postsynaptic response. B: signal remaining after glutamate receptor blockage corresponded to presynaptic current. N1a was almost unaffected by application of 1 mM Ni2+, whereas N1b was reduced. Tracing at bottom, Ni2+-sensitive current (control-Ni2+). C: both N1 and N2/SN were reduced by reducing the Ca2+/Mg2+ ratio from 2 mM/1.2 mM to 0.1 mM/3.1 mM (the balance of divalent cations was maintained). D: N2/SN, but not N1, were reduced by 10 μM Cl-adenosine. P1 did not change during pharmacological manipulations.

FIG. 3. Focal recordings from visualized mossy fiber terminals. A: mossy fiber and its terminal visualized with fast tetramethylindocarbocyanine perchlorate (DiI). Inset, terminal at higher magnification; calibration bars, 4 μm. B: stimulating and recording pipettes were positioned with Nomarsky optics on the points where the fluorescent image revealed a mossy fiber and its terminal (profile is redrawn from A and overlaid). Single tracings from terminal show a prominent inward current. Terminal current was not measurable after small displacement of the recording pipette from the terminal. Plot at right shows sharp dependence of the positive peak of the terminal current to changes in stimulation intensity. C: terminal current was reduced by perfusion with 1 mM Ni2+. Subtracting Ni2+ from the control tracing yielded the current, depending on Ca2+ channel activation (control-Ni2+).
postsynaptic response was investigated with specific glutamate receptor antagonists. To block NMDA receptors, we co-applied the glutamate site antagonist APV and the glycine site antagonist 7-CI-kyn, the combination of which has been shown to improve NMDA EPSP inhibition in whole cell recordings (D’Angelo et al. 1995). Figure 2A shows that application of 100 µM APV and 50 µM 7-CI-kyn left N₂ almost unaffected while reducing SN by 80.1 ± 16.8% (n = 8). The subsequent application of 10 µM NBQX (n = 8) reduced N₂ by 88.4 ± 19.3% (n = 8). N₂ and SN thus reflected postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptor activation in granule cells (Armano 2000; D’Angelo et al. 1995, 1999; Garthwaite and Brodbelt 1989).

The presynaptic current, which was isolated by ionotropic glutamate receptor blockade, was reduced by 41.7 ± 9.3% by perfusing a non-specific Ca²⁺ channel blocker, 1 mM Ni²⁺ (Fig. 2B). In those cases in which N₁b could be separated from N₁a, N₁b was significantly more reduced than N₁a (−43.7 ± 7.5% versus −9.5 ± 7.3%; n = 6; P < 0.01 by paired t-test). The sensitivity of N₁a and specifically of N₁b to Ca²⁺ channel blockers, suggests that the presynaptic response includes a component originating from presynaptic terminals (see below and Figs. 3C and 8A). P₁/N₁a was blocked by TTX, thus corresponding to the axon volley (data not shown).

The functional relationship of N₁ with neurotransmitter release was clarified by using three complementary manipulations. First, reducing the Ca²⁺/Mg²⁺ ratio is known to reduce presynaptic Ca²⁺ influx and neurotransmitter release (Dodge and Rahamimoff 1967). When the Krebs solution (1.2 mM Ca²⁺/1.2 mM Mg²⁺) was substituted with one containing a 50-fold lower Ca²⁺/Mg²⁺ ratio (0.1 mM Ca²⁺/3.1 mM Mg²⁺), N₁, N₂, and SN were all considerably reduced (−53.3 ± 10.4%, −49.4 ± 8.2%, and −70.4 ± 18.4%, respectively; n = 3). Similar results were obtained with the use of two Ca²⁺ channel blockers, Ni²⁺ and ω-CgTC-MVIIC (see Fig. 8B). Consistently, N₁, N₂, and SN increased when K⁺ channels were blocked by tetraethylammonium (TEA; see Fig. 9), a manipulation known to cause a secondary Ca²⁺ current enhancement (Angaut-Pettit et al. 1989; Brigant and Mallart 1982; Mallart 1985).

Second, activation of adenosine receptors is known to reduce neurotransmitter release at several peripheral and central synapses (Fredholm 1995). In these experiments we used Cl-adenosine (Fig. 2D), a rather selective antagonist of the A₁ receptor subtype, which is usually located presynaptically and is abundantly expressed in the cerebellum. As 10 µM Cl-adenosine was perfused into the bath, N₁ remained unchanged, whereas N₂ and SN decreased (−0.1 ± 17.2, −27.7 ± 5.0, and −72.1 ± 45.7%, respectively; n = 3). Although adenosine receptors might enhance K⁺ and inhibit Ca²⁺ channels (reviewed in Fredholm 1995), the changes observed here are in keeping with the early proposal that adenosine receptors affect release through a metabolic pathway independent of transmembrane Ca²⁺ influx (Silinsky 1984).

Third, during PPS (20-ms interpulse interval), which is known to regulate neurotransmitter release by influencing vesicle turnover and presynaptic Ca²⁺ accumulation rather than Ca²⁺ influx (Regher and Tank 1991; Wu and Saggau 1994), N₂ and SN increased, whereas N₁ remained stable (see Fig. 7). Thus although neurotransmitter release was influenced through different mechanisms, only those involving a regulation of Ca²⁺ influx were associated with a presynaptic current change.

Focal recordings from visualized mossy fiber terminals

The emergence of the terminal current into N₁ was directly demonstrated by recordings from an extracellular patch-pipette positioned on cerebellar mossy fiber terminals visualized with fast DiI (Fig. 3, A and B). Thirty to sixty minutes after positioning a dye crystal on the mossy fiber bundle, individual mossy fibers and their terminals were clearly distinguishable with fluorescence optics. The mossy fiber terminals showed a typical digitated shape, with smallest and largest diameters of 4.0 ± 1.2 and 5.2 ± 1.4 µm (cf. Eccles et al. 1967; Palay and Chan-Palay 1974).

After stimulation of the afferent axon, recordings from visualized terminals showed a triphasic extracellular current with a major negative peak. The current had three notable properties. First, it arose in an all-or-none manner by increasing the stimulus intensity (Fig. 3B, right). The presence of a nonzero signal at low stimulus intensities may indicate either electrotonic spread of subthreshold depolarization along the afferent axon or activation of neighboring axons with a lower activation threshold. Second, the current vanished after a few micrometers of pipette displacement outside the terminal. Finally, the current inward peak occurred at 1.2 ± 0.4 ms (n = 6; Fig. 3C) and was reduced by 45.9 ± 21.4% (n = 5) by 1 mM Ni²⁺. These observations are consistent with recordings from a single mossy fiber terminal with marginal contribution, if any, of other excitable elements. The terminal current showed timing and Ca²⁺ sensitivity identical to N₁ or N₁b, confirming that composite waveforms included a sizeable component generated by mossy fiber terminals.

LTP is associated with a presynaptic current increase

LTP was induced by TBS of the mossy fiber bundle (Armano et al. 2000; D’Angelo et al. 1999; Hansel et al. 2001). After TBS, both N₂ and SN increased signaling potentiation in non-NMDA and NMDA receptor-dependent responses, respectively. Strikingly, the presynaptic current also increased after TBS, whereas no changes were observed in control recordings (Fig. 4A).

N₁ and N₂/SN potentiation developed along a similar time course (Fig. 4B). Thirty minutes after TBS, N₁, N₂, and SN were enhanced by 38.1 ± 31.9, 49.6 ± 48.8, and 59.1 ± 35.5%, respectively (n = 7). The relationship between N₁ and N₂ amplitude changes 30 min after TBS is shown in Fig. 4C. Regression over the data shows a linear correlation of N₁ over N₂ (P < 0.05 by F-test), indicating that the presynaptic change reflects the intensity of LTP.

During LTP, P₁ did not change (−5.5 ± 31.8%; n = 7). Moreover, when N₁a could be separated from N₁b (Fig. 4D), N₁b increased significantly more than N₁a (35.4 ± 9.2 vs. 3.0 ± 13.0%; P < 0.01 by paired t-test; n = 3). The increase in N₁b, compared with the stability of P₁ and N₁a, points to a specific enhancement in the terminal current.

Presynaptic current increases in mossy fiber terminals

A direct demonstration of the origin of presynaptic current changes was obtained in recordings from visualized mossy
fiber terminals (Fig. 5A). After TBS, the terminal current showed an increase similar to that measured in N1 (35.2 ± 15.8%; n = 3).

It should also be considered that Purkinje cell discharge may be altered after repeated parallel fiber and climbing fiber activation causing N1 to increase after TBS (Eccles et al. 1967; Hansel et al. 2001). However, this possibility was ruled out by inducing LTP in experiments in which Purkinje cell activation was prevented by either removing the Purkinje cell layer (IFScut) or using TFS (Fig. 1). With IFScut and TFS, we obtained robust LTP both in N1 and N2/SN (Fig. 5B), and a specific N1b increase could be observed in four of nine experiments (e.g., Fig. 5B, inset). A slight reduction in the intensity of changes compared with those obtained with IFS probably reflected lower stimulation efficiency, because N1 and N2/SN were all similarly reduced.

Manipulations that prevent LTP also prevent the presynaptic current increase

If the presynaptic current increase depends on LTP induction, then it should be prevented by the same manipulations previously reported to block cerebellar mossy fiber–granule cell LTP in patch-clamp recordings (Armano et al. 2000; D’Angelo et al.

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**FIG. 4.** Simultaneous pre- and postsynaptic current increase during long-term potentiation (LTP). A: LTP was induced by θ-burst stimulation (TBS) in IFS experiments. Recording stability was monitored in experiments of the same duration but without TBS. Tracings show focal currents 20 min after stimulation began (thin line, just before TBS in LTP experiments) and 30 min thereafter (thick lines, 30 min after TBS in LTP experiments). Note that both pre- and postsynaptic currents increased after TBS but not in recordings without TBS. Plots show time course of N1, N2, and SN relative amplitude changes in experiments with TBS (at the arrow; filled symbols) or without TBS (open symbols). Data points are means ± SE from 7 experiments. B: examples of N1 and N1b potentiation following TBS in 2 different recordings. Note stability of P1 in both cases. C: relationship between N1 and N2 amplitude changes. Data were obtained from experiments in A (▲, Fig. 5B (●), and Fig. 8A (Δ)). Line shows linear regression over the data (n = 19; P < 0.05 by F-test).
N1 and N2 showed changes similar to those observed during LTP induced with 1999). Indeed, both N1 and N2/SN increases were prevented when bicuculline was omitted from the extracellular solution to cause granule cell inhibition through the Golgi cell circuit, thereby preventing removal of Mg2+ block from NMDA receptors (Fig. 6A). Moreover, both N1 and N2/SN increases (Fig. 6B) were prevented by blocking NMDA receptors with 100 µM APV and 50 µM 7-Cl-kyn, type 1 metabotropic glutamate receptors (mGlur-R-1) with 15 µM AIDA (Shoepf et al. 1999), and PKC with 10 µM GÖ 6983 (Bortolotto and Collingridge 2000). Neither changes could be observed in N1b when it could be isolated (e.g., Fig. 6A, inset). It should be noted that neither AIDA nor GÖ 6983 caused any remarkable changes in basal neurotransmission; the nature of inhibitory waves disclosed by omitting bicuculline and the changes caused by APV and 7-Cl-kyn are shown in Figs. 1 and 2.

These observations, in addition to confirming that voltage-dependent NMDA receptor activation and the mGlur-1-inositol trisphosphate (IP3)-PKC pathway (Masgrau et al. 2001) are involved in cerebellar mossy fiber–granule cell LTP, demonstrate that N1 (or N1b) changes are not generated by high-frequency stimulation itself but depend on efficient LTP induction.

Presynaptic currents are unchanged during PPS

Postsynaptic responses to PPS, by revealing the short-term depressing and/or facilitating properties of a synapse, have been largely used to shed light on the mechanism and locus of neurotransmission changes during LTP (Schultz et al. 1994). Here, in addition, we have measured the effect of PPS on the presynaptic terminal current (Fig. 7A). During PPS at 50 Hz (Fig. 7B), the N2 ratio \([N_{2(2nd)} - N_{2(1st)}]/N_{2(1st)}\) was \(-60.1 \pm 10.0\%\) (n = 4), reflecting a prevalence of synaptic depression. No comparable changes were observed in the N1 ratio \([N_{1(2nd)} - N_{1(1st)}]/N_{1(1st)} = -9.9 \pm 5.1\%\) (n = 4). The slightly negative value in the N1 ratio may reflect a contribution of terminal current reduction to short-term depression (Forsythe et al. 1998). During LTP (Fig. 7B), the N2 ratio increased, whereas the N1 ratio remained constant. Thus during LTP, the second pulse in a pair was facilitated without corresponding changes in terminal currents.

Pharmacological properties of presynaptic current changes during LTP

If the presynaptic current increase occurs in the mossy fiber terminal, then the N1 component sensitive to Ca2+ channel blockers would be specifically enhanced. Indeed, in experiments in which a 5-min, 1 mM Ni2+ perfusion was performed before and after LTP induction (Fig. 8A), Ni2+ block of N1 during LTP was enhanced (50.1 ± 22.6 vs. 31.4 ± 20.2%; P < 0.006 by paired t-test; n = 4). A similar result was obtained with a peptidic Ca2+ channel blocker, 5 µM ω-CTx-MVIIC (Meir et al. 1999). Because ω-CTx-MVIIC block is almost irreversible, different recordings were used for control and LTP experiments. Application of 5 µM ω-CTx-MVIIC caused a significantly larger N1 reduction 30 min after LTP induction than occurred in control recordings (42.2 ± 7 vs. 27.9 ± 4.6%; P < 0.01 by unpaired t-test; n = 4). It should be noted that N2 was also reduced by ω-CTx-MVIIC and Ni2+ and that this reduction was enhanced during LTP (with both blockers, P < 0.02; n = 4; Fig. 8B).

Finally, we considered whether LTP could be occluded by manipulating presynaptic terminal excitability. To this end, we applied 1 mM TEA, which has been shown to enhance depo-
larization and neurotransmitter release at central synapses (Lærum and Storm 1994; Mallart 1985; Meir et al. 1999). TEA application reversibly enhanced both N1 and N2 (69.7 ± 25.3 and 74.6 ± 15.0%; n = 6), thus mimicking the changes observed during LTP. In the presence of TEA, neither presynaptic nor postsynaptic currents were enhanced by TBS (N1 and N2 changes were -4.1 ± 12.5 and -1.1 ± 6.9%; n = 5) (Fig. 9). It should be noted that potentiation induced by TEA differed from K+ channel-dependent LTP (Anizkstein and Ben-Ari 1991; Zakharenko et al. 2001) because TEA had a low concentration, its effect was reversible (data not shown), and it did not determine neuron burst discharge.

**DISCUSSION**

In this study, we show that extracellular focal recordings provide a means to perform noninvasive, long-lasting pre- and postsynaptic measurements of mossy fiber–granule cell LTP. Our main observation is that during LTP, the presynaptic terminal current persistently increased. The relevance of this finding is related to the ability to identify the terminal current and measure it simultaneously with the postsynaptic response, allowing a direct relationship between pre- and postsynaptic processes to be established. Before addressing the potential mechanism of presynaptic current potentiation and its implications for LTP expression, we will consider the nature of recorded signals.

**FIG. 6.** Pharmacological blockage of LTP and presynaptic current changes: application of TBS in IFS experiments in different pharmacological conditions. Tracings were taken just before TBS (thin lines) and 30 min thereafter (thick lines). Plots show time course of N1, N2, and SN relative amplitude changes (mean ± SE). A: absence of bicuculline (●; n = 6). Inset, separation of N1a and N1b.

B: perfusion of 100 μM APV and 50 μM 7-Cl-kyn (○; n = 5), 15 μM AIDA (△; n = 5), or 10 μM GO 6983 (□; n = 4). Note the absence of potentiation (or even a tendency to depression for ●, ○, and △) in all experimental conditions.

The focal current reflects excitation at the mossy fiber–granule cell relay

The focal current recorded from the granular layer of cerebellar slices after mossy fiber stimulation could be interpreted based on current knowledge of anatomical, functional, and pharmacological properties of the cerebellar circuitry (Eccles et al. 1967; Garthwaite and Brodbelt 1988; Palay and Chan-Palay 1974). The presynaptic response (P1/N1) fell within the synaptic delay (<2 ms), and the postsynaptic response (N2/SN) fell in correspondence with intracellular EPSP-spike complexes of granule cells (D’Angelo et al. 1995). In particular, N2 was a fast non-NMDA receptor-mediated component priming an action potential, and SN was a slow NMDA receptor-mediated component. Golgi cells controlled the amplitude of N2/SN through a large GABA A receptor-mediated hyperpolarizing wave, the effectiveness of which decreased with the temporal separation between excitation and inhibition. These observations indicate that the mossy fiber–granule cell relay, which has a large numerical and volumetric prevalence over other circuit elements of the granular layer, was the main source of the focal current.

Both the pre- and postsynaptic components of the focal current were usually graded with stimulation intensity, indicating recruitment of mossy fibers and transition of granule cell responses from subthreshold EPSPs to spike firing (see Fig. 1A). Peculiar to focal recordings is that the mossy fiber termi-
nal current emerged from $N_1$ as a specific $Ca^{2+}$-sensitive component, which could be separated as a distinct peak ($N_{1b}$) in ~50% of cases and could be measured from single mossy fiber terminals made fluorescent with DiI. The small size and close temporal contiguity of $N_{1b}$ relative to $N_{1a}$, combined with small phase differences in axonal and terminal excitation, can easily explain the blurring of $N_{1b}$ into $N_1$ in some recordings.

The influence of circuit elements other than the mossy fiber–granule cell relay, in particular Purkinje cells, was ruled out by using TFS, by surgically removing the Purkinje cell layer, and by activating inhibitory interneurons to prevent Purkinje cell activation. The lack of Purkinje cell signals in focal recordings obtained from the granular layer is explained by two considerations. First, focal electrodes collected currents generated from a restricted area underneath their tips (Brigant and Mallart 1982; Del Castillo and Katz 1956; Forti et al. 1997; Katz and Miledi 1965; Mallart 1985; see also Fig. 3), being therefore unable to detect signals generated by Purkinje cells. Second, the granular layer of cerebellar slices receives Purkinje cell axons from a thin strip rather than from the overlying cerebellar surface. Thus the contribution of Purkinje cells to granular layer signals is strongly limited in slices compared with in vivo cerebellar recordings (Eccles et al. 1967). Golgi cells discharged too late to influence $N_1$, and their spontaneous activity was usually not observed, ruling out their potential contribution to focal currents in present experiments.

**LTP at the mossy fiber–granule cell relay**

LTP was induced by TBS of the mossy fiber bundle and, characteristically, included a robust potentiation in the presynaptic terminal current. The presynaptic change was $Ca^{2+}$-dependent, as expected from terminal but not axonal currents, and no changes occurred in the presynaptic volley. Moreover, the presynaptic change could be directly revealed in visualized
mossy fiber terminals and was not influenced by Purkinje cell discharge. The specificity of presynaptic changes for LTP was demonstrated by their absence when pharmacological manipulations caused LTP induction failure. Moreover, presynaptic changes were absent during short-term plasticity elicited by PPS or when neurotransmitter release was modulated through adenosine receptors (see Terminal current regulation and the mechanism of LTP expression).

LTP showed properties consistent with those measured with patch-clamp recordings from granule cells (Armano et al. 2000; D’Angelo et al. 1999). Although the present experiments did not allow us to control the locus of drug application, the preventative action exerted by synaptic inhibition and NMDA receptor blockers is consistent with a voltage-dependent control of granule cell NMDA receptor-dependent LTP induction through the Golgi cell circuit. Moreover, neither the mGluR-1 blocker AIDA (Schoepp et al. 1999) nor the PKC inhibitor GÖ 6983 (Bortolotto and Collingridge 2000) significantly affected basal neurotransmission, tending to exclude their presynaptic action. Consistently, the mGluR-1-IP$_3$-PKC pathway has been functionally and biochemically characterized in cerebellar granule cells (Aronica et al. 1993; Masgrau et al. 2001), where it has been proposed to influence LTP induction (D’Angelo et al. 1999; Rossi et al. 1996). It should also be noted that synaptic inhibition, APV, and AIDA, but not GÖ 6983, turned the effect of TBS toward long-term depression (see Bliss and Collingridge 1993; Fig. 6). This may reflect the presence of a PKC-dependent switch in the metabolic pathways involved in long-term synaptic plasticity (Bortolotto and Collingridge 2000), although incomplete PKC inhibition by GÖ 6983 or the contribution of other kinases cannot be ruled out.

LTP expression showed potentiation in both non-NMDA and NMDA receptor-mediated responses. LTP was more intense than in voltage-clamp but comparable to current-clamp recordings, probably reflecting a simultaneous increase in neurotransmitter release and intrinsic granule cell excitability (Armano et al. 2000; D’Angelo et al. 1999). LTP persisted for the entire duration of the recordings (usually 1 h) but could be measured for 6 h in five experiments (data not shown). This confirms the long-lasting nature of potentiation, the observation of which was usually limited to <1 h by using patch-clamp recordings from granule cells.

**Mossy fiber terminal excitation**

To reconstruct the process of presynaptic terminal excitation, one should recall that a depolarizing current runs upward if it is generated below the electrode or downward if it comes from neighboring regions. Thus invasion of the terminal by a depolarizing current coming from the axon causes the first downward deflection, P$_1$ (Brigant and Mallart 1982). This activates an inward current in the terminal, which largely depends on Ca$^{2+}$/H$_{9275}$-sensitive current in control experiments (a-b) and during LTP (d-e). Plots, time course of N$_1$ and N$_2$ changes (mean ± SE; n = 4); bars, Ni$^{2+}$ perfusion. B: application of 5 μM ω-CTx-MVIIC or 1 mM Ni$^{2+}$ caused a reduction in both N$_1$ and N$_2$. Histogram shows N$_1$ and N$_2$ block before and after LTP induction (mean ± SE; n = 4). SN showed changes similar to those in N$_2$.
However, the postsynaptic response changed independently from the terminal current after activation of A₁ adenosine receptors, in keeping with the cAMP-mediated reduction in Ca²⁺ sensitivity of vesicular release observed at the neuromuscular junction (Silinsky 1984) and in the rat hippocampus (Lupica et al. 1992). The postsynaptic response changed independently from the terminal current, also during PPS, which modifies the number of available vesicles and their release probability (see Lupica et al. 1992; Regher and Tank 1991; Schultz et al. 1994). Thus terminal currents were functionally related to neurotransmitter release through their Ca²⁺-dependent component.

During LTP, the Ca²⁺-dependent terminal current increased. Occlusion by TEA suggests that this was due to a K⁺ current reduction (Kandel and Schwartz 1982), raising Ca²⁺ relative to K⁺ currents. Thus a simple hypothesis is that during LTP, an increased Ca²⁺ influx raises neurotransmitter release (Hawkins et al. 1993; Ishikawa and Takahashi 2001; Kuba and Kumasato 1990). A presynaptic Ca²⁺ accumulation seems less probable (Regher and Tank 1991; Wu and Saggu 1994) because the paired-pulse facilitation ratio of the presynaptic response did not change with LTP. Moreover, the volley was stable and retrograde plasticity enhancing presynaptic neuron excitability (Ganguly et al. 2000) could not take place because the soma of neurons where mossy fibers are generated was not included in the slice preparation.

During LTP, the correlation of pre- and postsynaptic changes supports a causal relationship between terminal current and neurotransmitter release, and the change in paired-pulse facilitation of the postsynaptic response suggests a presynaptic locus of expression (Schultz et al. 1994). The hypothesis that neurotransmitter release is enhanced bears several mechanistic implications, including an increase in the frequency of minis, a decreased failure rate, and a decreased excitatory postsynaptic current variability (Bekkers and Stevens 1990; Bliss and Collingridge 1993; Hawkins et al. 1993; Malenka and Nicoll 1999; Malgaroli and Tsien 1992). Because presynaptic changes depend on LTP induction in granule cells, a retrograde messenger has to diffuse backwards to the mossy fiber terminal to cause the presynaptic change (Schuman and Madison 1991). Nitric oxide, which is released by granule cells on NMDA receptor stimulation (Garthwaite et al. 1988), might play this role.

We point out that, although the properties of presynaptic current changes consistently support a presynaptic locus, we cannot exclude that postsynaptic changes such as an increased intrinsic excitability (Armano et al. 2000; Hansel et al. 2001) or receptor expression (Malenka and Nicoll 1999) could also contribute to mossy fiber LTP expression.

Conclusions

Our results indicate that, similar to what has been observed in invertebrates, changes in presynaptic terminal excitability occur in certain vertebrate synapses during long-term synaptic plasticity (Hawkins et al. 1993; Kandel and Schwartz 1982). This observation, together with evidence of increased vesicle cycling (Nayak et al. 1996; Zakharenko et al. 2001), reinforces the hypothesis that presynaptic changes enhance neurotransmitter release during LTP. However, it is unknown whether these mechanisms take place at all brain synapses or whether

Terminal current regulation and the mechanism of LTP expression

Both the terminal current and the postsynaptic response changed conjointly when Ca²⁺ influx was modified by altering the Ca²⁺/Mg²⁺ ratio or by blocking Ca²⁺ or K⁺ channels.

FIG. 9. Occlusion of LTP and presynaptic current changes by K⁺ channel blockers. Involvement of K⁺ channels was investigated by 1 mM tetraethylammonium (TEA) application. After a control recording period (a), application of 1 mM TEA increased N₁ and N₂ (b). TBS (arrow) could not induce any change in N₁ or N₂ (c). Plots, time course of N₁ and N₂ changes; bar, TEA perfusion (mean ± SE; n = 3). SN showed changes similar to those in N₂.
they subserve specific regulatory actions. We surmise that presynaptic current plasticity can simultaneously regulate transmission to the numerous granule cells impinging on each mossy fiber terminal (28 on average in the rat; see Fig. 1C) (Eccles et al. 1967). This would contribute to spatial processing of mossy fiber discharge in the cerebellar granular layer and to motor control (Eccles et al. 1967; Medina and Mauk 2000).

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