Synaptically Evoked Glutamate Transport Currents May Be Used To Detect the Expression of Long-Term Potentiation in Cerebellar Culture

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Linden, David J. Synaptically evoked glutamate transport currents may be used to detect the expression of long-term potentiation in cerebellar culture. *J. Neurophysiol.* 79: 3151–3156, 1998. Cerebellar long-term potentiation (LTP) is a use-dependent increase in the strength of the granule cell-Purkinje neuron synapse that occurs after brief stimulation of granule cell axons at 2–8 Hz. Previous work has shown that cerebellar LTP also may be seen when synaptic currents are evoked in granule cell-glial cell pairs in culture. This finding suggests a model in which cerebellar LTP is expressed presynaptically and therefore may be detected by either neuronal or glial postsynaptic cells. However, synaptic currents evoked in both granule cell-glial cell pairs and granule cell-Purkinje neuron pairs in culture are mediated primarily by $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors, raising the possibility that cerebellar LTP might be expressed postsynaptically in both glial cells and Purkinje neurons in a similar manner. To address this question, glutamate transport currents were recorded in granule cell-glial cell pairs in culture by pharmacological isolation. These currents were increased by substitution of internal Cl with NO$_3$, and were blocked by 1-pyrrolidine-2,4-dicarboxylate, both characteristics of the major cloned Bergmann glial cell glutamate transporter, EAAT1. After acquisition of baseline responses, LTP of isolated transport current was evoked by stimulation at 4 Hz (100 pulses) and could be blocked by removal of external Ca during this stimulation. The expression of LTP was associated with a decrease in the rate of synaptic failures and a decrease in the degree of paired-pulse facilitation. These findings, when taken together with the previous observation that both Purkinje neuron and glial AMPA/kainate responses can be used to detect cerebellar LTP, strongly suggest that the expression of cerebellar LTP is, at least in part, presynaptic. This strategy should also be useful in illuminating the locus of expression of other model systems of information storage such as hippocampal LTP/long-term depression.

INTRODUCTION

It has been suggested that use-dependent modification of the strength of the parallel fiber-Purkinje neuron (PN) synapse in the cerebellar cortex is necessary for certain forms of motor learning including associative eyelink conditioning and adaptation of the vestibuloocular reflex. One cellular model system that has been examined as a candidate mechanism for such information storage is cerebellar long-term depression (LTD), in which coactivation of climbing fiber and parallel fiber inputs to a Purkinje neuron induces a persistent, input-specific depression of the parallel fiber-Purkinje neuron synapse (see Linden and Connor 1995 for review). The converse phenomenon, cerebellar long-term potentiation (LTP), also has been described, in which the parallel fiber-PN synapse is strengthened by repetitive parallel fiber stimulation at low (2–8 Hz) frequencies (Crepel and Jaillard 1991; Hirano 1990, 1991; Sakurai 1987, 1990; Salin et al. 1996; Shibuki and Okada 1992), thus endowing this synapse with the important capacity of use-dependent bidirectional modification.

Several lines of evidence have suggested that the induction of cerebellar LTP requires presynaptic Ca influx. Application of glutamate receptor antagonists during the tetanic stimulation is ineffective in blocking cerebellar LTP induction (Linden 1997; Salin et al. 1996). However, cerebellar LTP may be induced when the PN is loaded with a Ca chelator (Linden 1997; Sakurai 1990; Salin et al. 1996; Shibuki and Okada 1992) but not when external Ca is removed during tetanic stimulation (Linden 1997; Salin et al. 1996). One potential mechanism by which an increase in presynaptic Ca could be linked to LTP expression is the activation of a Ca-sensitive adenylate cyclase, such as the type I isoform of adenylate cyclase, which is enriched in cerebellar parallel fibers, and the consequent activation of adenosine 3',5'-cyclic monophosphate (cAMP)-dependent protein kinase. In support of this model, it has been shown that cerebellar LTP may be blocked by an inhibitor of cAMP-dependent protein kinase and that a LTP-like effect may be produced by an adenylyl cyclase activator (Salin et al. 1996). Furthermore, cerebellar LTP, but not a LTP-like effect produced by a cAMP analogue, is strongly attenuated in granule cell-PN pairs prepared from mutant mice which lack the type I adenylate cyclase (B. Hacker, C. Hansel, A. Parent, D. Storm, and D. Linden, unpublished observations).

Several findings have been proposed in support of the hypothesis that the locus of cerebellar LTP expression is presynaptic as well. First, it is associated with a decrease in the rate of synaptic failures when measured in granule cell-PN pairs (Hirano 1991; Linden 1997). Second, it is associated with a decrease in paired-pulse facilitation when studied in the cerebellar slice (Salin et al. 1996). Unfortunately, neither of these results are definitive (see Kullmann and Siegelbaum 1995; Wang and Kelly 1997 for a discussion of similar issues in relation to the locus of expression of hippocampal LTD). Third, it was shown that activation of a cerebellar granule neuron can give rise to a rapid inward current in an adjacent glial cell that is ~90% mediated by activation of $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors and ~10% by electrogenic glutamate reuptake. Stimulation of the granule neuron (4 Hz × 100 pulses) can give rise to LTP of the glial synaptic current, which has properties indistinguishable from those of granule cell-Purkinje neuron LTP (Linden 1997). These findings suggest a model in which cerebellar LTP is ex-
pressed presynaptically and therefore may be detected by either neuronal or glial postsynaptic cells. However, an alternative explanation is that cerebellar LTP is expressed post-synaptically via mechanisms that are common to Purkinje neurons and cerebellar glia, presumably those that would alter the function of AMPA/kainate receptors in both cell types.

If cerebellar LTP expression is presynaptic, then it should be detectable not only by recording Purkinje neuron and glial AMPA/kainate receptor-mediated synaptic responses but also by recording that fraction of the glial synaptic current that is mediated by electrogenic glutamate reuptake. This presents a technical problem, however, as this current comprises only ~10% of the total current in this preparation, thus making it very small when recorded in isolation. Fortunately, a solution to this problem has been provided by the observation that the anion channel component of the glutamate reuptake current may be potentiated by substitution of internal Cl (the dominant endogenous permeant anion) with anions of a higher chaotropic number such as NO3 or SCN. This has been demonstrated using glutamate transporter currents evoked by exogenous glutamate in heterologously expressed cloned glutamate transporters, including EAAT1, the species that predominates in cerebellar Bergmann glia (Wadiche et al. 1995). In addition, this manipulation has been performed with exogenous glutamate in retinal glia cells (Eliasof and Jahr 1996) as well as synaptically evoked transporter currents recorded in cerebellar Purkinje neurons (Otis et al. 1997), cerebellar Bergmann glia (Bergles et al. 1997; Clark and Barbour 1997), and hippocampal astrocytes (Bergles and Jahr 1997) in slices. In the present study, pharmacologically isolated, synaptically evoked glutamate transporter currents are recorded in NO3-loaded granule cell-glial cell pairs to determine if they can be used to detect the expression of cerebellar LTP.

METHODS

Embryonic neurons and glia from mouse cerebellum were prepared and cultured according to the method of Schilling et al. (1991). Whole cell recordings were made from glial cells (9–15 DIV) as previously described (Linden 1997; Linden et al. 1991). Cells were bathed in a solution that contained (in mM) 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, and 0.02 picrotoxin, adjusted to pH 7.35 with NaOH, which flowed at a rate of 0.5 ml/min. The recording electrode contained (in mM) 110 CsCl, 10 tetraethylammonium (TEA)-Cl, 10 HEPES, and 10 Cs4-bis-(o-aminophenoxy)-N,N′,N′,N′-tetraacetic acid (BAPTA), adjusted to pH 7.35 with CsOH. Where indicated, CsCl was substituted with CsNO3. Patch electrodes were pulled from N51A glass and polished to yield a resistance of 3–5 MΩ when measured with the internal and external salines described above. They were attached to glial somata and were used to apply a holding potential of ~80 mV. For stimulation of granule neurons, slightly smaller electrodes (5–8 MΩ) were fabricated. These electrodes were attached to the
FIG. 3. Long-term potentiation (LTP) of synaptically evoked transport currents in granule cell-glial cell pairs. A: after acquisition of a baseline transport current response in NO3-loaded cells held at -80 mV, LTP was induced by 4 Hz × 100 pulse stimulation (indicated by horizontal bar at t = 0 min). Each data point represents a single synaptic response. LTP was induced by 4 Hz × 100 pulse stimulation (indicated by heavy horizontal bar at t = 0 min). B: amplitudes of synaptically evoked reuptake currents after induction of LTP in a population of granule cell-glial cell pairs, n = 6 cells. Current traces are the average of 10 consecutive responses from a single cell at the times indicated. Scale bars = 4 ms, 10 pA. Stimulus artifacts have been truncated. C: glial transport current responses were abolished by nominal removal of Ca in the external saline (light horizontal bar at t = -7.5 min). After 4 Hz × 100 pulse stimulation (heavy horizontal bar at t = 0 min), Ca was replaced to restore transporter currents and assess their amplitude, n = 5 cells. Current traces are the average of 10 consecutive responses from a single cell at the times indicated. Scale bars = 5 ms, 10 pA. D: after 4 Hz × 100 pulse stimulation in the absence of external Ca, normal external Ca was restored revealing a failure to induce LTP. A second 4 Hz × 100 pulse stimulation delivered to the same cell in the presence of external Ca (heavy horizontal bar at t = 20 min) was successful in inducing at least the initial stages of LTP.

Granule neuron soma with negative pressure and were driven by a constant voltage stimulus isolator to evoke an action potential in the granule neuron. Test pulses were applied at 0.1 Hz. Membrane currents were recorded with an Axopatch 200A amplifier in resistive voltage-clamp mode, filtered at 2 kHz and digitized at 5 kHz using an Instrutech ITC-16 interface and Axodata software (Axon Instruments). Experiments were conducted at room temperature. 1-pyridinyl-2,4-dicarboxylate (1-PDC), d-2-amino-5-phosphono-pentanoic acid (d-AP5), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were obtained from Tocris and all other compounds from Sigma.

RESULTS

Glial cells were identified in culture as large cells with a stellate or fusiform shape and nontapering processes, which had resting potentials less than -75 mV (when recorded with K-based saline), input resistance of <30 MΩ (when recorded with Cs/TEA-based saline), and no spiking on injection of depolarizing current. In some cases, this identification was confirmed with immunocytochemistry using an antiserum directed against glial fibrillary acidic protein (see Linden 1997). After establishment of whole cell patch-clamp recording from a glial cell, a second patch electrode was pressed against a neighboring granule cell and a brief (0.05-0.25 ms) constant voltage pulse (50-200 V) was applied to evoke an action potential in the granule neuron. This process was repeated until a monosynaptically connected (latency =5 ms) granule cell-glial pair was found, at which point suction was applied to the stimulating electrode to form a tight seal on the membrane of the granule cell.

Pharmacological isolation of glutamate transport currents was achieved by adding the following drugs to the external saline: d-AP5 (100 μM), to block N-methyl-d-aspartate receptors, CNQX (30 μM), to block AMPA/kainate receptors, (+)-α-methyl-4-carboxyphenylglycine (MCPG; 500 μM) to block
metabotropic glutamate receptors, bicuculline (100 μM) to block γ-aminobutyric acid-A (GABA_A) receptors, and 2-OH saclofen (200 μM) to block GABA_B receptors. The remaining glial current was very small when recorded with a Cl-based internal saline (4.3 ± 2.0 pA, mean ± SE, n = 9 cells, V_hold = −80 mV). In an effort to increase the size of this current to the point where it could be measured more easily, a NO_3-based internal saline was used to increase the anion conductance mediated by the glutamate transporters (Wadiche et al. 1995). This allowed for the clear resolution of failures and successful synaptic transmission, the latter of which had a mean amplitude of 45.8 ± 16.5 pA (n = 8 cells, V_hold = −80 mV). Three lines of evidence suggest that this synaptic current is mediated by electrogenic glutamate transport (Fig. 1). First, the increase in current amplitude by substitution of Cl for NO_3 is a signature that, to my knowledge, is unique to the glutamate transporter-associated anion channel. Second, the current-voltage relation of the isolated synaptic current in NO_3-loaded glial cells shows that it does not reverse, even at very positive values of V_hold consistent with a transporter action (Wadiche et al. 1995). Third, the isolated glial synaptic current is attenuated ~10-fold by application of the glutamate transport inhibitor L-PDC (300 μM) (Bridges et al. 1991) but not by octanol (200 μM), a blocker of gap junctional coupling (Bernardini et al. 1984). Taken together, these findings strongly suggest that the synaptic current measured in these conditions is mediated predominantly by electrogenic glutamate transport.

To determine whether the synthetically evoked transporter current can detect changes in the probability of release, an agonist of the adenosine A1 receptor (2-chloroadenosine, 1 μM) was added to the bath while granule cell-glial cell transporter currents were evoked with test pulses (Fig. 2). This manipulation produced an attenuation of the mean evoked transporter current (51 ± 5.4% of baseline at t = 10 min, mean ± SE, n = 4) that was associated with an increase in the synaptic failure rate from 42 ± 7.3% at t = −5 min to 66 ± 8.0% at t = 10 min. 2-Chloroadenosine has been shown previously to decrease the probability of release as measured in the parallel fiber-Purkinje cell synapse in a slice preparation through an attenuation of voltage-gated Ca channel function in the presynaptic terminal (Dittman and Regehr 1996).

Because induction of cerebellar LTP does not require the activation of glutamate receptors (Linden 1997; Salin et al. 1996), it should be possible to induce LTP in the conditions described above for pharmacological isolation of glutamate transporter currents (Fig. 3A and B). Baseline synaptic transporter current responses were recorded in NO_3-loaded glial cells when the paired granule cell was stimulated at 0.1 Hz. This resulted in a mixture of synaptic currents and failures that were averaged to produce an index of synaptic strength (mean reuptake current amplitude). After baseline acquisition, LTP was induced by 4 Hz × 100 pulse stimulation, after which test pulses were resumed. Induction of LTP was accompanied by a significant decrease in the failure rate (49 ± 7% at t = −2.5 min before LTP induction compared with 23 ± 9% at t = 20 min after LTP induction, n = 6) and a significant increase in the mean amplitude of evoked reuptake currents (178 ± 10% of baseline at t = 20 min, n = 6). The amplitude of this form of LTP was comparable with that previously measured (Linden 1997) using granule cell-Purkinje neuron synaptic currents (193 ± 24% of baseline at t = 20 min, n = 6) or granule cell-glial cell synaptic currents in the absence of drugs (186 ± 16% of baseline at t = 17.5 min, n = 7), which are dominated by AMPA/kainate receptor-mediated responses.

Because cerebellar LTP has been shown to be blocked when external Ca is removed during the induction stimulus, this manipulation was performed together with a LTP induction protocol applied to isolated glial glutamate transport currents. Nominal removal of external Ca resulted in a rapid abolition of synthetically evoked transport current (2 ± 4% of baseline at t = 0 min, n = 5), at which point 4 Hz × 100 pulse stimulation was applied and after which Ca was restored. After equilibration of the Ca-containing external saline, it was revealed that no significant potentiation had been induced (94 ± 6% of baseline at t = 20 min; Fig. 3C). To assure that removal and return of normal external did not produce trauma to the cell pair that might have prevented the expression of cerebellar LTP, a separate experiment was performed in which a second 4 Hz × 100 pulse stimulation was applied at t = 20 min, when normal external Ca had been restored (Fig. 3D). This treatment was capable of inducing (at least the earliest phase of) cerebellar LTP at that time (150% of baseline at t = 27.5 min).

Previous work using the cerebellar slice preparation has shown that the induction of cerebellar LTP is associated with a decrease in paired-pulse facilitation of parallel fiber excitatory postsynaptic currents (EPSCs) (Salin et al. 1996). This finding has been offered as evidence in favor of a presynaptic locus of expression (but see Wang and Kelly 1997). To determine whether a similar alteration of paired-pulse facilitation occurs in LTP of isolated glial glutamate transporter currents, the LTP induction protocol described above was repeated in conditions where test pulses were replaced with pulse pairs (40-ms interpulse interval). To provide an accurate index of paired pulse facilitation, 30 successive responses were averaged, and the ratio of the second to the first response was calculated for this averaged response. Before LTP induction, paired-pulse facilitation

FIG. 4. LTP of synthetically evoked transport currents is associated with a decrease in paired-pulse facilitation. Test pulse pairs were applied at an interval of 40 ms. Paired-pulse facilitation was measured as the ratio of the 2nd pulse amplitude to the 1st pulse amplitude for averages of 30 consecutive stimuli. LTP was induced by 4 Hz × 100 pulse stimulation (heavy horizontal bar at t = 0 min). Each point represents the mean ± SE of 5 cells.
was 238 ± 9% (t = −10 to −5 min, n = 5). After induction of LTP, this value was reduced to 172 ± 16% (t = 20 to 25 min).

**DISCUSSION**

The main finding of this report is that synaptically evoked glutamate transport currents may be recorded using granule cell-glial cell pairs in culture, and these currents may be used as detectors of cerebellar LTP induced by tetanic stimulation. LTP of glial transport currents may be induced in the presence of postsynaptic Ca chelation, glutamate receptor antagonists, and voltage clamp of the postsynaptic cell but is blocked by removal of external Ca during the tetanus. Furthermore, LTP of glial transport currents is associated with a decrease in paired-pulse facilitation. These properties of cerebellar LTP are similar to those previously reported for the AMPA/kainate receptor-mediated EPSC recorded in PNs in slice (Crepel and Jaillard 1991; Sakurai 1990; Salin et al. 1996; Shibuki and Okada 1992) and culture (Hirano 1990, 1991; Linden 1997) preparations, as well as the cultured glial synaptic current recorded in the absence of drugs, which is mediated predominantly (~90%) by the AMPA/kainate receptors (Linden 1997). Although it remains formally possible to argue that cerebellar LTP is expressed in a similar manner by all three of the detectors of synaptic glutamate release mentioned earlier, a more parsimonious explanation is that cerebellar LTP is expressed, at least in large part, presynaptically.

The presence of synaptic currents in cerebellar glia that are mediated by a mixture of AMPA/kainate receptors and electrogenic glutamate transport is consistent with the reported immunohistochemical localization of both GluR1 (Bauda et al. 1994) and EAAT1 (Chaudry et al. 1995) to that portion of the Bergmann glial plasma membrane that wraps the synapses received on PN dendritic spines. Similar mixed glial synaptic currents have been reported in cultured hippocampal neurons (Mennerick and Zorumski 1994; Mennerick et al. 1996), Bergmann glia in the cerebellar slice (Bergles et al. 1997; Clark and Barbour 1997), and in hippocampal astrocytes in a slice preparation (Bergles and Jahr 1997). In these preparations, the proportion of synaptic current mediated by glutamate transport was higher than in the present case. It should be cautioned that because electron microscopy has yet to be performed in conjunction with the present recordings, it is unclear whether the glial currents recorded herein always represent spillover of transmitter involved in neuron-neuron communication. Similarly, because glia in culture adopt various morphologies, it cannot be determined which proportion of the glial cells recorded in culture derive specifically from Bergmann glia.

The use of NO₃ loading to reveal synaptically evoked glutamate transport currents should be applicable to determination of locus of expression for a number of use-dependent modifications of synaptic strength, including hippocampal and neocortical LTP and LTD. Recording of both glial and neuronal (Otis et al. 1997; Takahashi et al. 1996) transport currents will be instructive in this regard. However, one caveat that is worth sounding is that the physiological effects of NO₃-loading have not been systematically investigated and therefore run the risk of altering some aspects of neuronal and glial function. For example, NO₃ could be metabolized to peroxynitrite, which has been reported to inhibit glutamate transport (Trotti et al. 1996).

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