Temporal Specificity of Long-term Depression in Parallel Fiber–Purkinje Synapses in Rat Cerebellar Slice

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Abstract

The phenomenon of cerebellar long-term depression (LTD), a decrease of synaptic strength between the parallel fibers (PFs) and Purkinje cells after conjunctive activation of PFs and the climbing fibers (CFs), is implicated as a cellular mechanism for motor learning. We have characterized a field-potential recording technique in cerebellar slice and have used the technique to examine the temporal conditions for cerebellar LTD induction in an attempt to examine the relevance of LTD to associative conditioning. Interstimulus intervals (ISIs) between onsets of PF and CF activation and the number of paired stimuli (pairings) were examined. LTD has distinct temporal specificity that seems to be constrained by inhibitory interneurons and can be masked by excessive stimulation. When 100 paired stimuli were given to PFs and CFs, LTD was induced with an ISI of 250 msec (PF activation preceding CF activation). In contrast, a smaller forward (125 msec), simultaneous (0 msec), or backward (–250 msec) ISIs were not effective for inducing LTD. However, the blockade of GABA_A receptor-mediated inhibition made it possible to induce LTD with 100 pairs of simultaneous stimulations. Furthermore, by increasing the number of pairings to 600, significant LTD was observed with all four interstimulus intervals. These results suggest that

temporal conditions for LTD induction share some similarity to associative learning of discrete motor responses.

Introduction

One of the most fundamental questions in the broad field of neuroscience is how memories are formed and stored in the mammalian brain. In the cerebellum, synaptic transmission between the parallel fibers (PFs) and Purkinje cells undergoes long-term depression (LTD) after brief conjunctive stimulation of climbing fibers (CFs) and PFs (Ito et al. 1982; for reviews, see Ito 1989, 1993). The resulting LTD is associative because stimulation of either CF or PF alone does not produce LTD. Although this form of synaptic plasticity has been suggested to underlie some forms of motor learning (for reviews, see Thompson 1986; Ito 1987; Siegelbaum and Kandel 1991), lack of parametric studies of cerebellar LTD makes it difficult to evaluate systematically its relevance to behavioral learning (Thompson et al. 1993; Thompson and Krupa 1994). We have developed a simple but reliable recording technique to study the temporal conditions for LTD induction in the context of associative learning of discrete behavioral responses (e.g., eyeblink).

Recent rapid progress in research on hippocampal long-term potentiation (LTP) illustrates that field-potential recording in the brain slice is a valuable tool in studying long-term plasticity (Bliss and Lomo 1973; for review, see Bliss and Collingridge 1993). But difficulty in obtaining well-defined field potentials in the cerebellar slice, noted by Ito (1989), has been an obstacle in studying LTD. In the present study we have developed a field-potential recording technique in cerebellar slice and have been able to identify the components of the

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field potential in Purkinje cell dendrites evoked by parallel fiber activation. The population excitatory postsynaptic potential (EPSP), mediated mostly by AMPA receptors, is kinetically and pharmacologically well defined. We demonstrate that LTD can be reliably induced using field-potential recording in cerebellar slices. Considering its technical simplicity, temporal stability and its reflection of the population response, the *in vitro* field potential offers some advantages over previously used intracellular or whole-cell recordings for evaluating the temporal conditions for LTD induction.

Temporal specificity is perhaps the *sine qua non* of associative learning; it is the common feature of virtually all types of associative learning in all species studied (Hawkins et al. 1986; Gormezano et al. 1987; Hearst 1988; Rescorla 1988; Clark et al. 1994). Learning is optimal when the conditioned stimulus (CS) precedes the unconditioned stimulus (US) by some period of time (ranging from msec to sec to min, depending on the learning paradigm), consistent with the view that conditioning involves learning about predictive relationships in the environment. For classical conditioning of discrete motor responses, the optimal interval is between 200 and 400 msec and no learning occurs with simultaneous or backward pairing of the CS and US (Gormezano 1972; Thompson and Krupa 1994). Interestingly, classical conditioning of discrete movements in *Aplysia* exhibits a similar optimal CS-US interval (Clark et al. 1995). Here, we study the interstimulus interval (ISI) function in cerebellar LTD. We find that LTD induction has a distinct ISI function that appears to be constrained by inhibitory interneurons, a function that shares some similarity with associative learning of discrete motor responses. This synaptic temporal specificity may be explained by G protein-linked intracellular events underlying cerebellar LTD (for review, see Linden 1994). A preliminary report of these reports was presented earlier (Chen and Thompson 1992).

Materials and methods

RAT CEREbellar SLICE PREPARATION

Male Long-Evans rats (100–200 grams; Simonson Labs, Gilroy, CA) were housed on a 12-hr light–dark cycle and were anesthetized with methoxyflurane (Pitman-Moore, Mundelein, IL) in a desiccator prior to decapitation. As soon as the

skull was opened, icy oxygenated saline was poured over the brain. The cerebellar vermis was isolated from the two hemispheres and the rest of the brain.

The vermis was glued by a thin film of cyanoacrylate glue and was supported by a block of solid agar in a slicing chamber that was immediately filled with the ice-cold, oxygenated solution. Sagittal slices of 400- μ m thickness were made with a razor blade on a Vibratome. The tissue was cut with slow speed (0.5 unit) and maximal vibration amplitude (10 units) to ensure minimal damage. Both top and bottom sections were discarded; only five slices (midline plus two from each side) were used for electrophysiology. Slices were transferred to the recording chamber with a spatula to avoid any mechanical distortion.

Cerebellar slices were placed on a nylon mesh at the interface between a humidified atmosphere of 95% O₂/5% CO₂ and the oxygenated solution consisting of (mM): NaCl, 124; KCl, 2.5; MgCl₂, 1.3; CaCl₂, 2.5; NaH₂PO₄, 26.2; HEPES, 10; dextrose, 10. The pH was adjusted to 7.4 when necessary. Antagonists, CNQX (6-cyano-2,3-dihydroxy-7-nitroquinoxaline, from Tocris Neuramin, Bristol, England), bicuculline (Sigma Chemical Co., St. Louis, MO), or tetrodotoxin (TTX, from Sigma), were dissolved in the saline prior to application. The slices were constantly perfused at a rate of 30–60 ml/hr. All recordings were made at room temperature (22°C) after 1 hour of incubation.

ELECTROPHYSIOLOGY

Under the dissecting microscope, three layers of the cerebellar cortex, including the molecular layer, Purkinje cell layer, and granule cell layer, and the white matter could readily be identified in each folium. A recording glass microelectrode (1–3 M Ω), filled with 2 M NaCl, was positioned in the upper molecular layer, and right above, in the surface of a folium, a concentric bipolar stimulating electrode was positioned (Rhodes Medical Instruments, Woodland Hills, CA). Thus, a 0.1-msec voltage pulse through the stimulating electrode would activate the PFs, and evoke field potentials in the Purkinje cell dendrites. For paired stimulation, another bipolar electrode was usually placed in the white matter near the recording electrode to stimulate the CFs.

Field potentials were recorded with an AC amplifier (AM-Systems, Seattle, WA) and filtered with

cutoff frequencies at 0.1 Hz and 5 kHz. Data were acquired and analyzed in a personal computer using the pCLAMP programs (Axon Instruments, Foster City, CA). EPSP amplitudes instead of slope were measured to avoid any contamination by the PF volley. In some cases, EPSP slopes were also measured and found to correlate highly with the peak amplitudes.

TWO-PATHWAY RECORDINGS AND STIMULATION PARADIGM

Two populations of Purkinje dendrites were monitored simultaneously to independently assess both the induced LTD and the general state of the slice. A glass microelectrode, placed in the dendrites of Purkinje cells, was accompanied by a bipolar electrode on the edge of the folium to stimulate PFs and by another bipolar electrode in the white matter to stimulate CFs (Fig. 1). This arrangement constituted a paired pathway in which the two inputs converging on Purkinje cells could be coactivated with a given ISI. Another recording electrode was placed 300 μm lateral to the first electrode in the paired pathway and accompanied with its own PF stimulating bipolar electrode. The second recording electrode formed a control pathway. When one pathway was stimulated via PFs or CFs, only the corresponding electrode recorded the full field potential. The other electrode picked up only the artifact and a small fiber volley. Thus, there was little cross talk between the two recording electrodes.

The PF-evoked EPSPs of both paired and con-

trol pathways were monitored every minute during the entire experiments. The baseline responses were stabilized for at least 10 min before the PFs and CFs in the paired pathway were coactivated repetitively at 1 Hz with a chosen interval, and then the EPSPs of both pathways were sampled 60 min afterwards. If the change of the PF–Purkinje cell EPSP in the control pathway (300 μm lateral to the paired pathway) exceeded 20% of the pre-stimulation baseline, the slice was rejected as being not healthy. Ten percent of the slices were rejected using this criterion. Stimuli for both testing and pairing were voltage pulses of a 0.1 msec duration; their intensities were adjusted to be $\sim 70\%$ of maximal responses. Either 100 or 600 repetitions were used, and four ISIs, -250 , 0 , 125 , and 250 msec, were tested for their effectiveness to induce LTD.

Results

WAVEFORM AND DISTRIBUTION OF FIELD POTENTIALS IN CEREBELLAR SLICE

The field-potential recordings were made mostly in the molecular layer of folia IV, VI, VII, or VIII (Ito 1984). Those folia were chosen because their molecular layers are uniformly long and wide, advantageous for the two-pathway recordings employed. The length of PFs is 400 μm , running orthogonally to the section plane. PFs were stimulated with a bipolar electrode on the surface of a recorded folium. Thus, the area of PF stimu-

Figure 1: The schematic of two-pathway extracellular recording. Purkinje cells and three inhibitory interneurons are depicted in the sagittal section of the rat cerebellum. PFs run along the broken lines. The recording electrode in the paired pathway (R_p) was accompanied by a PF and a CF stimulating bipolar electrode, whereas the recording electrode (R_c) in the control pathway was accompanied only by a PF stimulating electrode.

lation is confined to the on-beam Purkinje cell dendrites. CFs were activated through white matter stimulation. We did not attempt to differentiate other responses to white matter stimulation including antidromic Purkinje cell spikes and disynaptic activation through mossy fibers and granule cells (Sakurai 1987).

There are at least three kinetically distinct components in the field potential that could be reliably evoked by PF activation (Fig. 2A). The initial transient phase is the result of the stimulus artifact because it follows the stimulation pulse and can be seen with stimulation anywhere relative to the recorded slice. The artifact is followed by a fast triphasic component. This component is

Figure 2: Pharmacology and waveform of field potentials evoked by PF stimulation. (A) CNQX (20 μ M, 30 min) antagonized the slow component, indicating AMPA receptor-mediated EPSP. Addition of TTX (0.5 μ M, 20 min) abolished the triphasic component, suggesting a Na^+ channel-mediated volley. (Left) The stimulus artifact. Scale bars, 0.6 mV, 5 msec. (B) The EPSPs are shown before and after CNQX at greater amplification. Scale bars, 0.2 mV, 5 msec. (C) Bicuculline (10 μ M) had little effect on the field EPSP. Scale bars, 0.1 mV, 5 msec. (D) The entire CF response was abolished by CNQX. Scale bars, 0.6 mV, 5 msec.

due to PF action potentials (volley); the conduction of action potentials along the terminating PFs yields a triphasic response. The last slower component is caused by PF–Purkinje cell synaptic transmission. The above analysis is similar to the waveform of the *in vivo* field potentials evoked by PF stimulation characterized by Eccles et al. (1967).

The above analysis was further confirmed by pharmacological studies of the field potential. The slow component is markedly diminished by a specific AMPA receptor antagonist, 20 μ M CNQX (% reduction $88.6 \pm 2.0\%$, $n = 10$, as assessed at the end of a 30-min CNQX perfusion), and the fast triphasic component is abolished by the voltage-gated sodium channel blocker, 0.5 μ M TTX. The only component left is the stimulus artifact. Thus, the triphasic component is the PF action potentials (volley) mediated by Na^+ channels and the last component is the PF-evoked population EPSP that is mediated by AMPA receptors (Fig. 2A,B). The focal stimulation to the PFs did not appear to evoke a distinct waveform of inhibitory response (Fig. 2C), as suggested previously by Eccles et al. (1967) and Crepel et al. (1981). But a small increase of the population spike and in neuronal firing was seen by application of 100 μ M bicuculline, indicating that a blockade of inhibition increased the excitability of Purkinje cells. In contrast to the PF response, the field potential evoked by CF stimulation could be abolished completely by 20 μ M CNQX (Fig. 2D). There was no fiber volley present in the CF response, as confirmed by application of TTX, suggesting that stimulation in the white matter evoked mostly CF–Purkinje cell response rather than the antidromic spike or mossy fiber–PF–Purkinje cell response.

The peak amplitudes of the population EPSP evoked in PF pathways are smoothly graded with increasing stimulus intensities (Fig. 3A). These graded PF–Purkinje cell responses have been described by numerous authors using the sharp electrode intracellular recording method (Llinas and Sugimori 1980a,b; Crepel et al. 1981) or the whole-cell patch recording method (Audinat et al. 1990; Perkel et al. 1990; Llano et al. 1991). It is difficult to measure precisely the slope of the field EPSP because its initial phase is often contaminated with the fast PF volley. Thus, we decided to measure EPSP peak amplitudes to avoid the contamination. To understand the current sources of the field potential, voltages were measured in three locations at different depths (Fig. 3B). The

Figure 3: Intensity series and spatial distribution of PF EPSPs. (A) Normalized peak amplitudes are smoothly graded with increasing stimulation intensity. Five example traces (*inset*) are field potentials evoked by PF stimuli of various intensity (9, 18, 27, 36, and 45 V). Scale bar, 0.2 mV, 5 msec. (B) The spatial distribution of field potentials evoked by PF stimulation. The largest response is found during the on-beam PF stimulation (0, 0). The recording sites were marked by two coordinates (x and y in μm) in reference to the PF stimulation point (*inset*). Peak amplitudes were normalized and plotted against the depth in each location.

depth, as defined by the sagittal thickness of the cerebellar slice, corresponds to distance along the parallel fiber track. When it is as close as possible to the bipolar stimulating electrode ($x=0$, $y=0$ coordinates), the recording electrode records the maximal EPSP (100%) at $\sim 300 \mu\text{m}$ from the top surface and the minimal EPSP on both the top and bottom surface (Fig. 3B). A similar but much smaller EPSP profile is seen with a recording electrode 100 μm away toward the cell bodies ($x=0$, $y=100$). Little activity is detected with the recording electrode 100 μm lateral to the bipolar electrode ($x=100$, $y=0$). Thus, near on-beam PF stimulation evoked the largest postsynaptic responses.

LTD CAN BE INDUCED BY 600 PAIRINGS AT ALL INTERVALS

No long-term changes of PF–Purkinje cell EPSP amplitudes was seen when either PFs or CFs alone were activated at 1 Hz for 10 min (Fig. 4); no noticeable changes in the efficacy of the synaptic transmission were seen after 600 stimuli to either PF or CF. Thus, nonassociative stimulation to Purkinje cells did not induce any long-term plasticity.

We choose a convention for determining the order of the paired stimulation in accordance with classical conditioning of discrete motor responses (Thompson 1986). The onset of PF activation is

Figure 4: No long-term changes were seen as a result of CF or PF activation alone. (A) PF alone. Scale bar, 0.2 mV, 5 msec. Note numerical increase in PF alone by 60 m. (B) CF alone. In both cases, 600 pulses, starting at 0 min, lasted for 10 min (1 Hz). The time course of the EPSP peak amplitude (percent of baseline) in both pathways was plotted.

the reference point in time. A forward pairing of 250 msec, for instance, means that CF activation follows PF activation by 250 msec, or CF stimulus precedes PF stimulus in the case of a backward pairing. Four ISIs, -250, 0, 125, and 250 msec, were examined to elucidate the temporal specificity of LTD.

When 600 pairings were used, LTD was effectively induced with all four ISIs tested. The simultaneous pairings (ISI=0) induced a robust depression in PF-Purkinje cell synapses ($-19.7\% \pm 4.5\%$, mean $\pm$ S.E.M. percent change

from the baseline measured 30 min after the paired stimulation, $n=20$, $P<0.001$, t -test) (Fig. 5A). A smaller forward ISI (125 msec), in which CF was activated 125 msec after PF stimulus, produced significant depression ($-17.3\% \pm 4.3\%$, $n=10$, $P<0.005$) (Fig. 5B). The forward ISI of 250 msec produced significant LTD ($-25.4\% \pm 4.7\%$, $n=18$, $P<0.001$) (Fig. 5C). The backward interval (ISI = -250 msec) was also effective for LTD ($-20.8\% \pm 5.4\%$, $n=18$, $P<0.002$) (Fig. 5D). Thus, the degree of LTD, measured 30 min after the pairings (600 at 1 Hz), did not depend on the

Figure 5: LTD was induced with 600 paired stimuli at 1 Hz. (A) Simultaneous pairings ($n=20$). Scale bar (for all traces), 0.2 mV, 5 msec. (B) A small forward ISI of 125 msec ($n=10$). (C) A forward ISI of 250 msec ($n=18$). (D) A backward ISI of -250 msec ($n=18$). Both pathways are shown. Examples are taken before and 60 min after paired stimulation for each ISI (insets).

temporal order of two convergent stimuli (Fig. 6). This flat ISI function generated by 600 pairings indicates that temporal specificity is not present.

To examine the spatial specificity of the induced LTD, we initially placed the control recording electrode at various distances from the paired pathway. We found that the control pathway was not affected by the paired stimulation when it was placed 300 μ m away from the paired pathway. In this case, the PF–Purkinje cell EPSPs in the paired pathway dropped transiently to 50% of the baseline during the paired stimulation, whereas a slight

decrease or no change was observed in the control pathway (Fig. 5). Upon termination of the paired stimulation, the PF–Purkinje cell EPSP amplitudes in the paired pathway recovered partially and remained depressed for 1 hr. Meanwhile, the field EPSP in the control pathway remained unchanged or slightly decreased (Fig. 6). No change in the control pathway indicates that the LTD was confined within a radius of 300 μ m of the paired stimulation point and that the cerebellar slices were vital and stable over the course (1 hr) of measured LTD. Thus, the LTD is spatially specific.

Figure 6: With 600 pairings, all four ISI (-250, 0, 125, and 250 msec) are equally effective for the induction of LTD. Temporal specificity, defined by a distinct ISI function, is absent here. The degree of LTD is expressed as percent change of baseline (mean $\pm$ C.E.M.) measured 30 min after onset. (A) Paired pathway; (B) control pathway; (C) A–B.

TEMPORAL SPECIFICITY IN LTD IS SEEN WITH 100 PAIRINGS

When 100 paired stimuli were given, however, the ISI became important for LTD. The forward ISI of 250 msec was still found effective in LTD induction in the paired pathway ($-25.6 \pm 4.7\%$, 30 min after paired stimulation, $n = 13$, $P < 0.002$) (Fig. 7A). In contrast, other intervals including a smaller forward ISI of 125 msec ($-8.2 \pm 5.3\%$, $n = 12$), the simultaneous ISI ($-4.2 \pm 6.3\%$, $n = 13$), and the backward interval of -250 msec ($0.4 \pm 5.3\%$, $n = 11$) failed to produce any significant synaptic changes (Figs. 7 and 8). Furthermore, synaptic responses in the control pathway are not significantly different from each other for the four ISIs. In sum, the degree of LTD, measured 30 min after 100 pairings, depends on the temporal intervals between two converged stimuli, that is, PFs and CFs (Fig. 9). By comparison, LTD induced by 600 pairings does not seem to correlate with ISIs. Thus, LTD is associative and pairing-specific, and the temporal specificity depends on the number of paired stimulations received by Purkinje synapses.

When the GABA_A receptor antagonist was applied to the slice, the simultaneous pairings (100) became effective in inducing robust LTD (Fig. 8). The LTD in the presence of bicuculline ($-19.4 \pm 6.5\%$, $n = 13$), expressed as the net changes of EPSP amplitudes between the paired and the control pathways, was significantly different from the net changes in the normal saline ($-4.2 \pm 6.3\%$, $n = 13$, $P < 0.05$). We noticed that the control pathway tends to increase or decrease gradually after simultaneous stimulation depending on the presence or the absence of bicuculline, so we combined relative changes between two

Figure 7: With 100 pairings at 1 Hz, LTD is induced only by the forward ISI (250 msec) but not other intervals. (A) A forward ISI of 250 msec ($n=15$). Scale bar, 0.2 mV, 5 msec. (B) A small forward ISI of 125 msec ($n=12$). (C) A backward ISI of -250 msec ($n=11$). Both pathways are shown. Examples are taken before and 60 min after paired stimulation for each ISI (insets).

pathways to control for the effects that are not caused by pairings but by bicuculline. Thus, it appears that synaptic inhibition mediated by GABA_A receptors prevents LTD with 100 simultaneous pairings.

Discussion

These experiments have demonstrated that cerebellar LTD induction is constrained by two temporal parameters, the ISI and the number of paired stimulations. Furthermore, the effect of the ISI depends on GABA_A inhibition. These findings provide a new insight into the possible role of cerebellar LTD in associative motor learning behavior.

First, we demonstrate that the in vitro field-potential recording may provide some advantages over other methods for studying conditions of cer-

Figure 8: One hundred pairs of simultaneous stimulation does not induce LTD in the normal solution, but it becomes effective in inducing LTD in the presence of the GABA_A receptor antagonist. (A) Without the inhibitor ($n=13$). Scale bar (for all traces), 0.2 mV, 5 msec. (B) In the presence of 200 μ M bicuculline ($n=13$).

ebellar LTD induction. The sharp electrode or whole-cell patch recording has been effective in revealing cellular mechanisms of LTD. For example, Sakurai (1987) confirmed with the intracellular technique the stimulation paradigms to induce cerebellar LTD, which were first described by Ito et al. (1982) with *in vivo* field-potential recording in the intact cerebellum. Later, Sakurai (1990) by intracellular recording and recently, Konnerth et al. (1992) by whole-cell recording demonstrated intracellular calcium involvement during LTD induction. However, both intracellular and whole cell patch recording methods have their limitations. They can only record from a single cell, rather than from a population of neurons. They may disturb the intracellular environment, thus affecting the physiological conditions for LTD induction. In practice, they are technically more demanding and seldom stable over hours. In contrast, the field-potential recording is technically easy and temporally stable and the field EPSP reflects population changes. Thus, the field recording method is a useful complementary tool for studying long-term synaptic plasticity in mammalian brain tissue.

Our analysis of field potentials in cerebellar slice by using specific receptor antagonists directly confirm previous *in vivo* studies of the cerebellar cortex (Eccles et al. 1967). Local stimulation of PFs in the intact cerebellum generates a triphasic positive-negative-positive wave followed immediately by a negative potential (also called N_3 wave) (Eccles et al. 1967). The triphasic wave represents a current source-sink-source typical of a compound action potential in a volume conductor, thus, a PF volley, as verified using current source-density analysis (Nicholson et al. 1978). The following negativity was shown to be the Purkinje cell EPSP by blocking synaptic transmission with manganese (Nicholson et al. 1978). We show that these two components are mediated by voltage-gated Na^+ channels and AMPA receptors, respectively. Our results are in agreement with recent studies with whole-cell patch recording methods (Audinat et al. 1990; Perkel et al. 1990; Llano et al. 1991). Other properties of Purkinje cells have been characterized with the sharp electrode intracellular recording (Llinas and Sugimori 1980a,b; Crepel et al. 1981).

The field EPSP peak amplitude being measured in the present study is a reliable indicator of excitatory synaptic transmission. The transition between the fast PF volley and the slow EPSP slope

takes place well before the peak of the EPSP, indicating that the peak is clearly not contaminated by the fiber volley, although the initial phase of the EPSP may be. The spatial distribution of PF-Purkinje cell EPSPs verifies that PF-Purkinje cell synapses are mostly in the superficial molecular layer, that is, distal Purkinje dendrites. A smaller EPSP amplitude is seen when the recording electrode is moved toward Purkinje cell bodies. The maximal EPSP is observed approximately half way along the PF tract (i.e., $\sim 250\text{-}\mu\text{m}$ depth), indicating that more synapses are activated there. The stimulated PF beam is restricted to within $100\text{ }\mu\text{m}$ lateral to the bipolar electrode because little activity is detected outside this region.

Figure 9: A distinct ISI function of cerebellar LTD is seen with 100 pairings. (A) The degree of LTD depends on the ISI in the paired pathway. (B) Changes in the control pathway. (C) The difference between two pathways.

In contrast to the widely used hippocampal slice preparation, however, field-potential recording in cerebellar slice should be interpreted with more caution. First, the length of PFs is limited by the thickness of sagittal cerebellar slices. Hence, the field postsynaptic potential is often affected by the PF volley. We avoided this problem by using only the field response with a clear distinction between the putative EPSP and the fiber volley. Second, numerous inhibitory interneurons may contribute to the postsynaptic potential. By examining the field EPSP in the presence and absence of the GABA_A inhibitor, we found that there was not a distinct component of the inhibitory response that might affect our measurement of EPSP amplitude. But the blockade of GABA_A receptors did make population spikes more visible, suggesting an increase in Purkinje cell excitability. Furthermore, we designed the two-pathway recording to minimize possible complications of field-potential recording in the cerebellar slice. In one pathway, the recorded Purkinje cell populations received temporally paired stimulation from both PFs and CFs, whereas in the control pathway 300 μ m away, Purkinje cells were monitored throughout experiments without the paired stimulation. This two-pathway recording allowed us to monitor independently those nonspecific variables that were not caused by the intended manipulation, such as the health of the slice during the course of experiments. In our opinion, it is necessary to use this type of control for studies of synaptic depression.

Second, we show that by using 100 paired stimulations at 1 Hz, only the forward ISI of 250 msec was found effective for LTD induction, whereas the other three ISIs (-250 , 0 , and 125 msec) were not effective. Previously, LTD was induced by pairing PFs and CFs simultaneously or with CFs preceding PFs by tens of milliseconds (Ito et al. 1982; Kano and Kato 1988; Ito 1989). However, it is difficult to establish the temporal order between PF and CF activation in previous studies, owing to the short interval between stimulus pairs. For instance, the frequency most used is 4 Hz (e.g., Sakurai 1987). In this case, the interval between stimulus pairs is 250 msec. A backward pairing of 20 msec (i.e., CF stimulation preceding PF stimulation in one paired trial, ISI = -20 msec) could also be interpreted as the forward pairing of 230 msec (i.e., PF stimulation preceding the next CF stimulation, ISI = 230 msec) when the end of one paired trial is compared with the beginning of the next trial. By using a lower frequency of

1 Hz, the order of the pairing becomes more evident. The forward ISI of 250 msec, for instance, is sufficiently different from the equivalent backward ISI, -750 msec. Thus, the ambiguity in arguing for the functional implications of cerebellar LTD may be better resolved. Incidentally, we note that a forward ISI of 200 msec was found effective in inducing LTD as indicated by a decrease of extracellular K⁺ concentration and an increase of nitric oxide (NO) release, which is thought to mediate LTD in the cerebellar slice (Shibuki and Okada 1991; no GABA_A antagonists were indicated in this study).

We also show that in the presence of bicuculline (200 μ m), LTD could be induced by 100 simultaneously paired stimulations, which normally failed to induce LTD. The inhibition mediated by GABA_A receptors appears to prevent the induction of LTD in the case of simultaneous stimulations (100). Consistent with this, a recent paper by Karachot et al. (1994) similarly found little evidence that relative timing of PF and CF stimulation was critical for LTD induction when picrotoxin (20 μ m) was present in the perfusate. The interaction between inhibition and excitation in the cortex may thus constrain the temporal specificity of long-term depression. This is not surprising because inhibitory interneurons in the cerebellar cortex outnumber Purkinje cells 25 to 1 (Llinas and Walton 1990), and previously, most investigators applied GABA_A antagonists in cerebellar slices to enhance the probability of obtaining LTD [e.g., 20–40 μ m of picrotoxin (Sakurai 1987); 2.5 μ m of bicuculline (Crepel and Jailard 1991)]. It is not clear which type of inhibitory interneuron, basket, Golgi, or stellate cells, is most influential in determining the temporal specificity of LTD. Detailed study of this inhibitory interaction requires a comprehensive biophysical model of the cerebellar circuitry.

Karachot et al. (1994) reported that LTD was optimal when the ISI was 0 msec (i.e., simultaneous ISI), but no LTD was seen when PF stimulus preceded CF stimulus by 10–100 msec (i.e., forward ISIs). In addition, smaller LTD was induced when PF stimuli were delayed after CF stimuli by as much as 2 sec (i.e., backward ISIs). These results seem to contradict our findings. However, there are two major differences between their study and ours that make direct comparison difficult. First, the GABA_A blocker, picrotoxin (20 μ m), was used throughout their study, whereas we did experiments both with and without the blocker

(Fig. 8). We did see LTD as a result of simultaneous pairings in the presence of the GABA_A blocker but not in its absence. In this sense, our result could partially explain theirs. We argue that the temporal specificity might be altered in experimental conditions such as blockade of GABA_A inhibition. To assess whether induction conditions of LTD resemble certain forms of motor learning, we feel strongly that one should evaluate LTD in conditions as close to in vivo physiology as possible. This is a crucial point in our study. Second, Karachot et al. (1994) did not seem to give a clear picture of temporal specificity. For example, in one experimental condition that seems most comparable with our paper, Karachot et al. (1994) varied PF–CF intervals from -100 msec to 0 (1 Hz; Fig. 6). The resulting LTD fluctuated so dramatically that 16% mean depression was observed with an interval of -80 msec, 5% LTD with a -70 -msec interval, but 20% LTD with a -60 -msec interval. It is hard to understand how such a wide range of changes in LTD could be caused by varying the ISI from -70 to -60 msec. We suspect that the variability in LTD might reflect the variability of their intracellular recording as well as the small sample size (5–6 cells).

In another study by Ekerot and Kano (1989), LTD was reported to be optimal when the ISI was between -125 msec and -250 msec (backward ISIs). They measured simple spikes with extracellular recording in test and control pathways (similar to our two-pathway recordings) in decerebrated rabbits. But in our opinion, changes of simple spikes might not necessarily reflect synaptic plasticity because the firing property of a neuron depends on neuronal excitability and inhibition as well as synaptic changes. Thus, the phenomenon described in their study, although interesting, might not directly address the temporal specificity of LTD. Moreover, when PF stimulus preceded CF stimulus by 5–20 msec (i.e., forward ISIs), the authors had a mixed result in which 4 cells out of 7 showed depression.

It is not known how inhibition and ISIs influence LTD induction. Most of the inhibition comes from off-beam PF stimulation (Eccles et al. 1966). Focal PF stimulation applied in our study does not appear to evoke a distinct waveform of GABA_A-mediated inhibitory responses. We speculate that simultaneous stimulation of two fiber systems may widen the area of stimulating current spread and thus recruit additional off-beam PFs, possibly activating inhibitory interneurons. The temporal win-

dow for LTD induction may also be dictated by interactions among various participating second messengers, many of which are linked to G protein-coupled metabotropic glutamate receptors (mGluRs) (Ito and Karachot 1990; Linden and Connor 1991; Aiba et al. 1994; Conquet et al. 1994; Shigemoto et al. 1994; for review, see Linden 1994). For example, if the activation of mGluRs occurs only in the PF synapses, as suggested by several authors (Aiba et al. 1994; Shigemoto et al. 1994), we speculate that G-protein activation may lead to a slow rise of second messengers; in contrast, calcium elevation controlled by CF activation may be relatively rapid. This temporal difference would be further amplified if a diffusible messenger like NO is generated in presynaptic PF terminals (e.g., downstream to mGluR activation) and interacts with calcium in the Purkinje neuron to induce LTD. Interestingly, the effect of stimulus asynchrony is also found in isolated cultured neuromuscular synapses (which are free from inhibitory interactions). Dan and Poo (1992) reported that the degree of depression in those synapses correlates positively with the intervals (10–250 msec) between two convergent inputs, whereas simultaneous coactivation has no effect.

In contrast to 100 paired stimulations, robust and stable LTD was observed with 600 PF stimuli paired with CF activation with all four ISIs. Excessive stimulation of 600 trials seems to overcome the effect of ISIs. The underlying mechanism is not clear. We speculate that there may be a threshold below which interactions among second messengers are temporally restricted. Excessive stimulation might result in overly abundant second messengers that surpass that threshold.

LTD revealed by field potentials is associative. Little changes in the PF–Purkinje cell synaptic efficacy were seen with either PF or CF activation alone. Repetitive PF stimulation at 1 Hz slightly potentiated the PF–Purkinje cell synapses, as shown by others (Ito et al. 1982; Sakurai 1987). During paired stimulation but not by PFs or CFs alone, the paired pathway also displayed short-term depression. One possible cause of this short-term depression may be the depletion of presynaptic transmitters. The LTD induced by paired stimulation was confined to within $300\ \mu\text{m}$ of the paired stimulation pathway. This spatial specificity may provide a physical basis for plasticity in a microzone, an elemental structure-functional unit of cerebellar cortex, similar to the ocular column in the cerebral cortex (Oscarsson 1976).

Our results suggest that the temporal specificity of LTD resembles that for eyeblink conditioning. Cerebellar cortex, in addition to deep nuclei, has been suggested to play a critical role in associative learning of the eyeblink response (for reviews, see Thompson 1986; Yeo 1991; Thompson and Krupa 1994). According to this model, the CS and the US converge on Purkinje cells through PFs and CFs, respectively. The optimal interval for learning is when the CS (through PFs) precedes the US (through CFs) by 250 msec, and the efficacy falls off when the interval is too long or too short (Gormezano et al. 1987). To correlate changes in Purkinje cells with eyeblink conditioning, Shreurs et al (1991) took slices of cerebellar lobule HVI from trained and untrained rabbits and reported that the majority of Purkinje cells from trained animals showed decreases in afterhyperpolarizations to intracellular depolarization relative to controls, implying an increase in excitability. Although this result seems contradictory to the direction of changes predicted by LTD, it did not address changes at the synaptic level. In our opinion, it is a vastly difficult task to sample *in vitro* only those cells modified in a particular manner by behavioral learning. In studies to date of learning-induced changes in the patterns of Purkinje neuron responses (i.e., in eyeblink conditioning), two basic types of changes are seen in the CS-US onset interval: a decrease in the frequency of simple spikes that precedes (by as much as 90 msec) and accurately predicts the occurrence of the behavioral conditioned response and a similarly timed increase in the frequency of simple spikes (Berthier and Moore 1986; Foy and Thompson 1986; Thompson 1990). These results clearly present a very significant sampling problem for *in vitro* studies on cerebellar tissue from trained animals. It is tempting to speculate that Purkinje neurons showing learning-induced decreases are from microzones representing the learned response (e.g., eyeblink) and those showing increases are from microzones representing other responses that must be suppressed. The mechanisms yielding both learning-induced decreases and increases in within-trial Purkinje neuron responses must ultimately be characterized.

Our study by no means indicates that LTD revealed by our recordings in the cerebellar vermis is generally applicable to learning-specific neurons. To study this issue directly, it will be necessary to select those neurons responsive to both the CS and US *in vivo* and monitor their excitability or

synaptic responses in the course of learning. In addition, careful attention should be paid to parametric differences between LTD and associative conditioning. For example, the intertrial interval for LTD is 1 sec rather than 30 sec, as in the case for behavioral learning; the brain slice preparation undoubtedly lacks some network components important for implementing a motor behavior.

Recent progress using gene knockout technology in mice lends some support to the correlation between LTD and eyeblink conditioning (for review, see Lisberger 1995). Aiba et al. (1994) reported that mice deficient for mGluR1 receptors, a subtype of metabotropic receptors previously implicated in LTD (Shigemoto et al. 1994), lack cerebellar LTD (also Conquet et al. 1994) and are partially impaired in eyeblink conditioning although other aspects of cerebellar structure and synaptic transmission seemed relatively intact. In addition, these mutant mice have severe impairments in motor coordination. In sum, our study provides a simple analogy between a form of synaptic plasticity like LTD and learning. It is but one step in our effort to comprehend the nature of the brain process underlying a simple form of associative memory involving discrete motor responses.

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