The expression of cerebellar LTD in culture is not associated with changes in AMPA-receptor kinetics, agonist affinity, or unitary conductance

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Cerebellar long-term synaptic depression (LTD) is a model system of neuronal information storage that is expressed postsynaptically as a functional down-regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. What properties of postsynaptic AMPA receptors are changed? Several lines of evidence argue against changes in AMPA-receptor kinetics. Neither LTD evoked in cultured granule-cell Purkinje cell (PC) pairs nor an LTD-like phenomenon evoked by phorbol ester application was associated with alterations in evoked AMPA receptor-mediated excitatory post-synaptic current (EPSC) or mEPSC kinetics. LTD produced by pairing glutamate pulses with depolarization was not altered by prior application of the desensitization-reducing compound cyclothiazide. Finally, rapid application of glutamate to lifted PCs revealed no significant alterations in AMPA-receptor kinetic properties after LTD induction. When this system was used to apply varying concentrations of glutamate, no alteration in AMPA-receptor glutamate affinity was seen after LTD induction. Finally, peak-scaled nonstationary fluctuation analysis was applied to estimate AMPA-receptor unitary conductance before and after LTD induction in a cultured cell pair, and this analysis too revealed no significant change. These results suggest that cerebellar LTD may be expressed solely as a reduction in the number of functional AMPA receptors in the postsynaptic density [Wang, Y.-T. & Linden, D. J. (2000) *Neuron* 25, 635–664].

In the cerebellum, long-term modifications in both synaptic strength and intrinsic neuronal excitability have been suggested to underlie certain forms of motor learning (1, 2). The most intensively studied of these phenomena has been long-term synaptic depression (LTD) of the parallel fiber-Purkinje cell (PC) synapse (generally referred to as "cerebellar LTD"). It is a persistent, input-selective attenuation that is produced when parallel-fiber and climbing-fiber inputs to a PC are briefly coactivated at low frequency. Recently, progress has been made toward defining the molecular events necessary for its induction. These include three initial postsynaptic events: mGluR1 activation, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-receptor activation, and Ca influx via voltage-gated Ca channels. These three signals seem to converge upon the activation of protein kinase C (PKC). In addition, inhibition of postsynaptic protein phosphatase activity through a cascade involving NO, cGMP, and cGMP-dependent protein kinase also may be important (1).

A molecular understanding of an expression mechanism for cerebellar LTD also is beginning to emerge. Cerebellar LTD may be detected by using exogenous test pulses of AMPA-receptor agonists such as glutamate, quisqualate, or AMPA in various preparations, including intact cerebellum (3), slices (4), and cell cultures (5). Furthermore, cerebellar LTD may be seen with AMPA-receptor agonist test pulses in preparations that lack functional presynaptic terminals (6–8). Thus, it seems as if cerebellar LTD expression involves some form of functional down-regulation of postsynaptic AMPA receptors. This down-regulation potentially could result from changes in several properties including AMPA-receptor unitary conductance (γ), kinetics, P_{open}, glutamate affinity, or the number/spatial distribution of receptors in the postsynaptic membrane. Support for this last idea has come from experiments showing that postsynaptic manipulations that interfere with clathrin-mediated endocytosis block the induction of cerebellar LTD in culture, whereas manipulations that induce postsynaptic internalization of AMPA produce an LTD-like effect that mutually occludes LTD induced by coactivation (9). Furthermore, application of an exogenous PKC activator to produce an LTD-like effect results in internalization of AMPA receptors in the dendrites of cultured PCs (10). The processes that link PKC activation to AMPA-receptor internalization are not completely understood, but the phosphorylation of Ser-880 in the carboxyl-terminal tail of the AMPA-receptor subunit GluR2, the subsequent unbinding of GRIP (11, 12), and the recruitment of PICK1 (12) to this domain seem to be necessary for the expression of cerebellar LTD (13).

Although now there is evidence to suggest that reduction in the number of postsynaptic AMPA receptors comprises at least a portion of the expression mechanism for cerebellar LTD, there is no evidence that would exclude additional forms of AMPA-receptor modulation. The present study utilizes voltage-clamp recording in cell-culture preparations together with rapid agonist application to assess the potential alteration of AMPA-receptor kinetics, agonist affinity, and unitary conductance in the expression of cerebellar LTD.

Materials and Methods

Mouse embryonic cerebellar cultures were prepared as described (5). Cultures were maintained for 9–16 days *in vitro* before their use in patch-clamp experiments. For conventional whole-cell recording, patch electrodes attached to PC somata were filled with a solution containing 135 mM CsCl, 10 mM Hepes, 0.5 mM EGTA, 4 mM Na_2-ATP, and 0.4 mM Na-GTP, adjusted to pH 7.35 with CsOH. For perforated-patch recording (see Figs. 3 and 4), electrodes were filled with a solution containing 95 mM Cs_2SO_4, 15 mM CsCl, 8 mM $MgCl_2$, 10 mM Hepes, adjusted to pH 7.35 with CsOH. Electrode tips were filled with a small amount of this solution, and the shanks were backfilled with this solution supplemented with amphotericin B at a concentration of 300 μg/ml. Stable access resistance of <15 MΩ could be obtained within 10 min of gigaseal formation.

Cells were bathed in 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, 0.1 mM D-AP5, 0.0005 mM tetrodotoxin (TTX), and 0.3 mM picrotoxin, ad-

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Abbreviations: PC, Purkinje cell; LTD, long-term synaptic depression; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; γ, AMPA-receptor unitary conductance; PKC, protein kinase C; TTX, tetrodotoxin; EPSC, excitatory post-synaptic current; mEPSC, miniature EPSC; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline.

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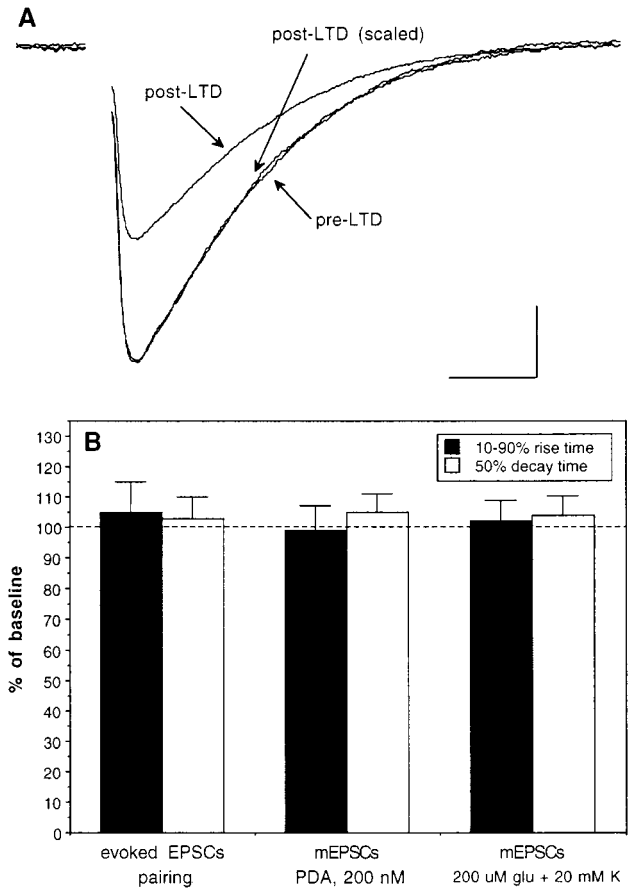

Fig. 1. LTD in cultured PCs is not associated with changes in the shape of either granule cell-evoked or miniature AMPA-EPSCs. (*A*) Evoked AMPA-EPSCs from a representative granule cell–PC pair (bathed in 0.1 mM D-AP5). Each trace is the average of 25 consecutive responses recorded starting 7 min pre- and 15 min postLTD. (Bars = 10 msec, 10 pA.) (*B*) Normalized kinetic measures from populations of cells that received either LTD induced by pairing granule-cell stimulation with depolarization (measured with granule cell-evoked EPSCs, $n = 6$) or an LTD-like phenomenon induced by bath application of phorbol-12, 13-diacetate (PDA; 0.2 μM at t = 0–15 min; measured with mEPSCs recorded in TTX, $n = 5$) or an LTD-like phenomenon induced by bath application of 200 μM glutamate plus 20 mM KCl (at t = 0–10 min; also measured with mEPSCs, $n = 5$)

justed to pH 7.35 with NaOH, which flowed at a rate of 0.5 ml/min. Patch electrodes were pulled from N51A glass and polished to yield a resistance of 2–4 MΩ. $V_{hold} = -80$ mV was imposed for experiments in which exogenous pulses of kainate or glutamate were applied. For recording AMPA-miniature EPSCs (mEPSCs), V_{hold} was set to −90 mV (to increase driving force). For the experiments shown in Fig. 2, iontophoresis electrodes (1-μm tip diameter) were filled with 10 mM glutamate or kainate (all in 10 mM Hepes, pH 7.1) and were positioned ≈20 μm away from large-caliber dendrites. Test pulses were delivered by using negative current pulses (600–900 nA, 30–110 msec duration). Two sets of experiments (Fig. 1, and see Fig. 5) used synaptic activation of granule cell–PC pairs, as described (9, 14). For these experiments, TTX was omitted from the external saline, and granule cells were activated by using 0.5-msec constant-voltage pulses through a loose-patch electrode (5–6 MΩ) filled with external saline. Rapid application of glutamate or kainate was achieved by using a piezoelectric positioner to laterally translate either a 2.0-mm diameter θ glass tubing (TGC200–15, Warner Instrument, Hamden, CT; see Fig. 3) or a custom-made linear six-barrel flowpipe (see Fig. 4) with streaming solutions delivered either by gravity or a syringe pump. The piezo device was driven by a series of brief ramps (total duration = 500 μsec) rather than a step function to reduce acceleration and consequent mechanical oscillation. The 20–80% solution exchange times for θ glass application were 1743 ± 215 μsec for whole cell and 380 ± 56 μsec for open tip recording modes (mean ± SEM; $n = 10$).

Estimates of γ were made by using peak-scaled nonstationary fluctuation analysis (15–17). This technique is based upon the assumption that the opening and closing of individual channels are independent events. The fluctuations in an event produced by the action of many channels will be smallest at the peak and the end of the event because most channels will be open at the peak and closed at the end. However, the intermediate amplitudes will show greater random fluctuations as determined by their open durations (resulting in a parabolic distribution for the graph of variance related to mean synaptic current). Also, the fluctuation of the synaptic current will depended upon the relative contribution of single channels to the total current: a small number of channels with large unitary conductance will produce larger fluctuations than a greater number of channels with small unitary conductance. In these experiments (see Fig. 5), TTX was removed from the external saline and the internal saline was supplemented with the Na channel blocker QX-314 (5 mM). For analysis, synaptic currents were aligned on the rising phase at the point of half-maximal amplitude, and an average waveform was calculated from 40 synaptic currents selected by eye to exclude failures and cases in which a second, spontaneous or polysynaptic current overlapped the first. Baseline variance was measured from the pre-event current. Peak-scaled variances then were calculated and the (weighted) mean single-channel current (i) and the number of single channels open at the peak of the synaptic current (N_p) were obtained by fitting the theoretical parabolic function below by using a least-squares algorithm:

$$\sigma^2 = iI - I^2/N_p + \sigma_b^2$$

In this equation, σ^2 is the variance accompanying synaptic-channel activity, I is the mean synaptic current, and σ_b^2 is the baseline variance. Unitary conductance (γ) was calculated from values of i derived from a fit of the initial slope of this relationship, together with the EPSC reversal potential as measured separately for each cell (−1 ± 2 mV, $n = 16$).

Membrane currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), and digitized at 10–20 kHz. Signals were low-pass filtered with an 8-pole Bessell filter at 5 kHz, except for the fluctuation-analysis experiments, in which an 8-pole Butterworth filter was used. Analysis was performed with AXOGRAPH 4.5 software. Recordings in which R_{input} or R_{series} varied by more than 11% were excluded from the analysis. TTX was from Alexis Biochemicals (San Diego), kainate and D-AP5 from Tocris Neuramin (Bristol, U.K.), phorbol-12, 13-diacetate from LC Laboratories (Woburn, MA), and QX-314 from Alomone Laboratories (Jerusalem). All other reagents were from Sigma.

Results

If cerebellar LTD were expressed in part as an alteration of AMPA-receptor kinetics, then this alteration should be reflected in changes in the shape of EPSCs after LTD induction. This idea was tested in two different ways. First, by using synaptically connected granule cell–PC pairs in culture, a baseline was collected with test stimuli applied at 0.1 Hz. This procedure evoked a mixture of EPSCs and synaptic failures. In this and all subsequent experiments, 0.1 mM D-AP5 was present in the external saline to block *N*-methyl-D-aspartate (NMDA)

receptors. LTD then was induced by pairing 60 granule cell stimuli, delivered at 0.5 Hz, each with a 50 msec PC depolarization to 0 mV, after which test pulses were resumed. Consistent with other observations (1, 9), this treatment produced an LTD of 62 ± 5% of baseline (mean ± SEM, measured at t = 20–25 min after LTD induction, $n = 6$). To assess EPSC kinetics, 25 EPSCs were averaged over the time period t = −10–0 min and again, after LTD induction, at t = 15–25 min. Scaled overlays of these averages from a representative cell reveal no alteration of EPSC kinetics (Fig. 1*A*), which is further reflected in population measures of 10–90% rise-time and 50% decay-time (Fig. 1*B*). This question also was addressed by using recordings of mEPSCs in TTX-containing saline. An LTD-like phenomenon may be produced by either brief bath application of PKC-activating phorbol esters (4, 10, 18) or by an external saline supplemented with 0.2 mM glutamate and 20 mM KCl (protocol modified from ref. 19). Application of phorbol-12, 13-diacetate (PDA, 200 nM; t = 0–15 min) to the external saline produced a significant depression of mEPSC amplitude (51 ± 7% of baseline; t = 25–30 min; $n = 5$) and frequency (81 ± 6%), but this depression was not associated with changes in the shape of mEPSCs (Fig. 1*B*). Similar results were found for the induction of an LTD-like phenomenon (amplitude, 56 ± 8% of baseline; t = 20–25 min; $n = 5$; frequency, 85 ± 5%) by a 10-min application of glutamate/KCl external saline.

Another test for the potential role of AMPA-receptor kinetics in cerebellar LTD expression used application of the desensitization-blocking drug cyclothiazide. If LTD expression requires an enhancement of the rate or extent of AMPA-receptor desensitization, then pretreatment with cyclothiazide should reduce the amplitude of LTD induction by blocking desensitization. These experiments were carried out with cultured PCs bathed in TTX and stimulated with iontophoretic test pulses of glutamate at 0.05 Hz. After 10 min of baseline recording, cyclothiazide (0.1 mM) was applied in the bath, which resulted in an increase in the amplitude of the glutamate-evoked inward current to 284 ± 12% of baseline ($n = 6$; Fig. 2*A*). After stabilization in cyclothiazide, the strength of the iontophoretic test pulse was reduced to match the baseline amplitude, after which LTD was induced by six 3-sec depolarizations to 0 mV, each paired with a glutamate test pulse (of the original baseline amplitude). The LTD evoked by this treatment was of an amplitude and duration that was indistinguishable from a control that did not receive cyclothiazide (cyclothiazide, 58 ± 8% of baseline, $n = 6$; control, 53 ± 7% of baseline, $n = 5$; both measured at t = 40–45 min). The reverse experiment also was performed, in which prior LTD induction failed to alter the subsequent response to cyclothiazide (Fig. 2*B*; LTD, 279 ± 15% of baseline, $n = 5$; control, 275 ± 17% of baseline, $n = 5$; both measured at t = 32.5–37.5 min). Finally, a related approach was taken in an experiment that used alternating test pulses of glutamate and kainate, the latter of which activates AMPA receptors in a weakly desensitizing fashion (Fig. 2*C*). After induction of LTD by glutamate/depolarization conjunction as described above, the amplitude and time course of LTD as revealed by glutamate and kainate test pulses was indistinguishable (glutamate, 53 ± 9% of baseline; kainate, 56 ± 7% of baseline; $n = 6$, t = 40–45 min).

Although iontophoretic pulses are a convenient way to deliver glutamate to cultured PCs, the kinetics of the currents that result are a complex mixture of many factors, including the turbulent flow of glutamate-containing solution over the surface of the PC. To assess directly the kinetics of AMPA receptors, a fast-flow system was used to deliver glutamate rapidly to cultured PCs recorded in perforated-patch mode that had been lifted carefully from the substrate. Similar to reported perforated dendritic macropatch recordings (7, 8), these preparations can express LTD as a result of glutamate/depolarization conjunction. Application of a 1.5-msec pulse of 1 mM glutamate produced an AMPA-receptor mediated inward current that approximated the shape of an EPSC (Fig. 3). This current was blocked by preincubation with 0.03 mM 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo-[*f*]quinoxaline (NBQX; data not shown) and could be reproducibly evoked if test pulses were repeated at intervals ≥60 sec. LTD was induced by 20 conjunctive stimuli, each consisting of a 100-msec glutamate pulse paired with coincident depolarization to 0 mV, delivered at intervals of 20 sec. This procedure resulted in a depression of the peak amplitude of the test pulse to 59 ± 7% of baseline when measured at t = 20–25 min ($n = 10$). When currents recorded −5–0 min before and 15–20 min after LTD induction were scaled and averaged, no significant alteration in kinetics could be seen. The shape of the glutamate-evoked current remained unchanged after LTD induction, as did the degree of recovery from desensitization produced when pulse pairs were delivered with an interval of 50 ($n = 5$) or 150 msec ($n = 5$). Taken together, the experiments shown in Figs. 1–3 argue against a role for changes in AMPA-receptor kinetics in cerebellar LTD expression.

Cerebellar LTD expression also could result from a reduction in the affinity of AMPA receptors for glutamate. To test this hypothesis, rapid application of agonist to lifted PCs was performed, but now a linear series of flowpipes, each loaded with a different concentration of glutamate, was used to construct a dose-response relationship. Each dose was applied for 50 msec, three times, in ascending concentration, with interpulse intervals of 60 sec (Fig. 4). Cyclothiazide (0.1 mM) was present throughout the experiment to block AMPA-receptor desensitization. LTD, evoked as described above, resulted in a decrease in the response to the highest dose of glutamate (10 mM) to 65 ± 10% of baseline (t = 20–30 min, $n = 7$). However, LTD was not accompanied by a significant change in the K_a (preLTD: 220 ± 24 μM, measured at t = −20–0 min; post LTD: 245 ± 31 μM, measured at t = 10–30 min, $n = 7$) or shape of the dose-response function. Thus, expression of cerebellar LTD in cultured PCs does not seem to involve a significant decrease in AMPA-receptor glutamate affinity.

Expression of cerebellar LTD might also result from a decrease in AMPA-receptor unitary conductance (γ). LTD was induced in synaptically connected granule cell–PC pairs in culture by pairing granule cell activation with depolarization of PCs, as shown (Fig. 1 *A* and *B*). For peak-scaled nonstationary fluctuation analysis, EPSCs were collected over the time period t = −15–0 min and again, after LTD induction, at t = 5–20 min. Cells were excluded from analysis if current rise or decay times were unstable or covaried with EPSC amplitude (16, 17) or potency (20). Furthermore, to select for synapses that were received more proximally and thereby reduce clamp error and dendritic filtering, cells were excluded from analysis if the 10–90% rise-time of the EPSC was longer than 2.5 msec (at room temperature). The cells that met these criteria showed an LTD of 58 ± 4% of baseline at t = 5–20 min after induction ($n = 10$; Fig. 5*A*). A representative current–variance plot of peak-scaled EPSCs collected before and after LTD induction is shown in Fig. 5*B*. The relationship is well fit by a parabolic function (see *Materials and Methods*) that yields an estimate of $\gamma = 21.6$ pA before LTD and 23.9 pA after LTD (coefficient of determination, $r^2 = 0.93$ and 0.94, respectively). When the population of 10 cells was analyzed, a similar result emerged, with $\gamma = 22.5 \pm 1.7$ pA before LTD and 23.3 ± 1.9 pA after LTD (Fig. 5*C*; $n = 10$). As a control, after acquisition of baseline responses in a protocol identical to that for the LTD experiment, the AMPA-receptor antagonist NBQX was applied at a concentration of 0.1 μM. This treatment reduced the EPSCs to 51 ± 7% of their initial value (at 5–20 min after NBQX application, $n = 6$). Similar to LTD induction, estimates of γ were unchanged: 23.1 ± 2.2 pA before NBQX and 23.4 ± 1.7 pA after NBQX (Fig. 5*C*;

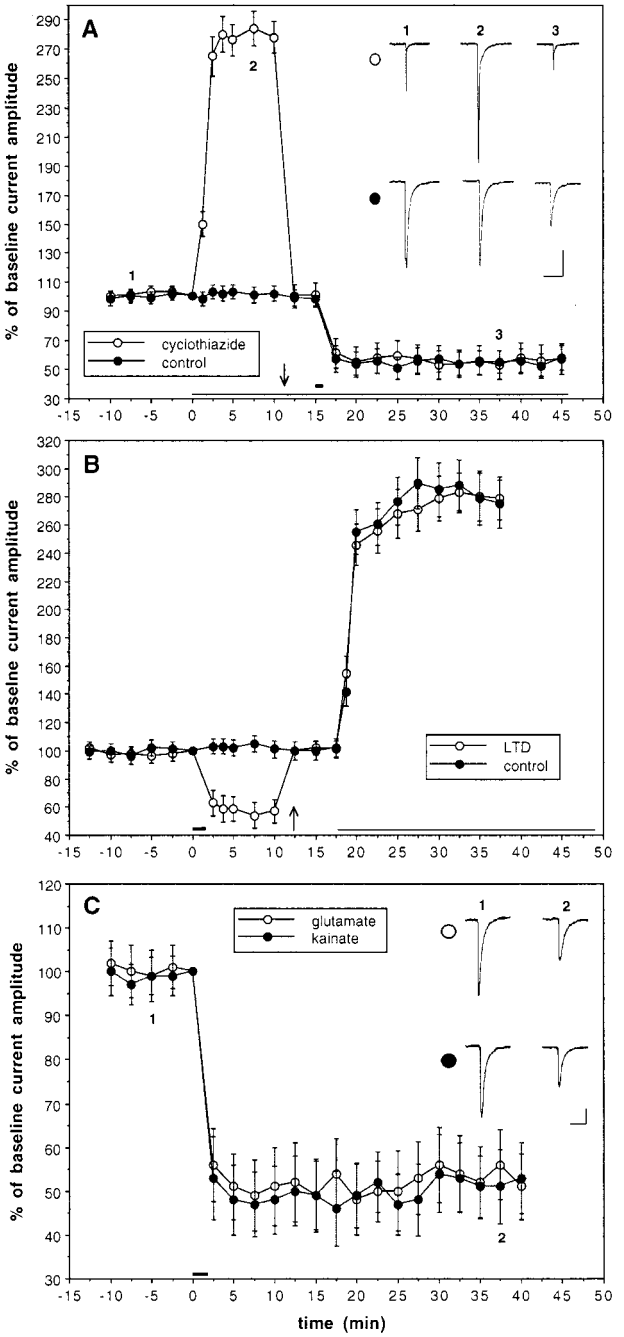

Fig. 2. Manipulations designed to alter AMPA-receptor desensitization have no effect on the expression of cerebellar LTD. (*A*) Cyclothiazide, a blocker of AMPA-receptor desensitization, does not affect subsequent LTD. Application of cyclothiazide starting at t = 0 min (0.1 mM, indicated by thin horizontal line above scale) produces a large potentiation of glutamate-evoked AMPA currents. After reduction in the duration of the iontophoretic test pulse to match baseline current amplitude (indicated by the downward arrow near t = 10 min), LTD was induced by glutamate depolarization conjunction (heavy horizontal bar at t = 15 min). (*Inset*) Single glutamate-evoked inward currents recorded in a representative PC at the times indicated. (Bars = 1 sec, 50 pA.) (*B*) The complementary experiment to that shown in *A*. Prior LTD induction (followed by software renormalization, indicated by upward arrow) does not affect the response to subsequent application of 0.1 mM cyclothiazide. (*C*) Alternating test pulses of glutamate and kainate (which is a weakly desensitizing AMPA-receptor agonist) were delivered to the same site on cultured PC dendrites before and after induction of LTD by glutamate/depolarization conjunction (heavy horizontal bar at t = 0 min). (*Inset*) Single glutamate- and kainate-evoked inward currents recorded in a representative PC at the times indicated. (Bars = 1 sec, 30 pA.)

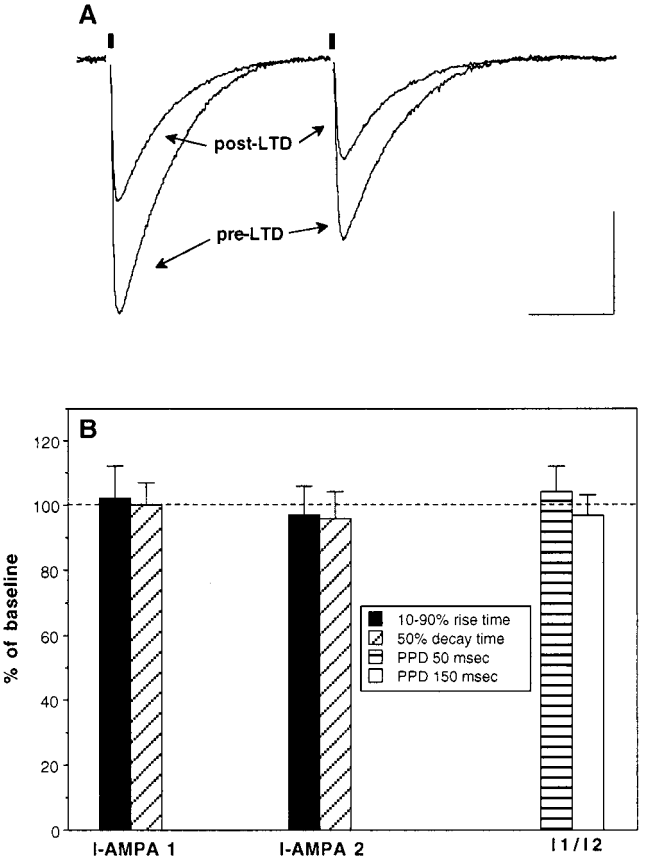

Fig. 3. Rapid delivery of glutamate with a fast-flow perfusion system fails to reveal changes in AMPA-receptor kinetics in lifted, cultured PCs. (*A*) Responses to 1.5-msec pulses of 1 mM glutamate (indicated by heavy vertical bars) in a representative PC were measured by using perforated-patch recording. Each trace is the mean of five consecutive responses. (Bars = 20 msec, 300 pA.) (*B*) Normalized kinetic measures from populations of cells that received LTD induction by glutamate/depolarization conjunction reveal no significant difference in AMPA currents evoked pre- or postLTD. A population of 10 PCs was divided into two groups of 5, which received paired-test pulses at intervals of either 50 or 150 msec. The 10–90% rise time and 50% decay time measurements are from pooled data, whereas the paired-pulse depression (PPD) measurement is shown separately for each group.

$n = 6$). These estimates are very similar to chord conductances derived from single-channel recordings in patches of somatic and dendritic membrane from cultured PCs that had not undergone LTD induction (D. Linden, unpublished data). In sum, LTD expression in granule cell–PC pairs in culture does not seem to be mediated by a decrease in AMPA-receptor γ.

Discussion

The main finding of these studies is that neither AMPA-receptor kinetics, agonist affinity, nor unitary conductance seem to be altered in cerebellar LTD expression. The first conclusion is supported by experiments showing that the kinetic properties of evoked EPSCs in granule cell–PC pairs are not altered after LTD induction. Similarly, the kinetic properties of mEPSCs are not changed after induction of an LTD-like phenomenon by exogenous PKC-activating phorbol ester or a glutamate/high K solution. In addition, LTD induced by pairing glutamate pulses with depolarization, was unaltered by pretreatment with the desensitization blocker cyclothiazide or by using test pulses of the weakly desensitizing agonist kainate. Finally, direct assessment of AMPA-receptor kinetic properties with a rapid application

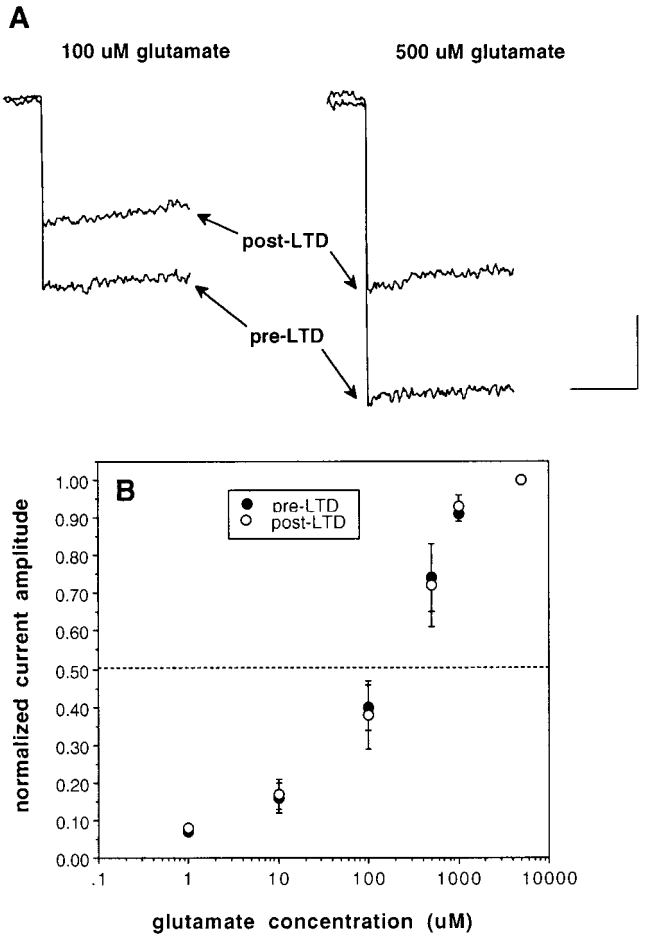

Fig. 4. A dose-response function shows no change in AMPA-receptor glutamate affinity after induction of LTD. (*A*) Representative single responses to doses of glutamate in a lifted, cultured PC measured −20–0 min before and 10–30 min after induction of LTD by glutamate/depolarization conjunction with perforated-patch recording. Cyclothiazide (0.1 mM) was present throughout the experiment to block AMPA-receptor desensitization. (Bars = 25 msec, 300 pA.) (*B*) A dose-response function relating mean glutamate-evoked current amplitude to glutamate concentration in seven PCs, before and after LTD induction. Current amplitudes have been normalized to the maximum response in each group (pre- vs. postLTD).

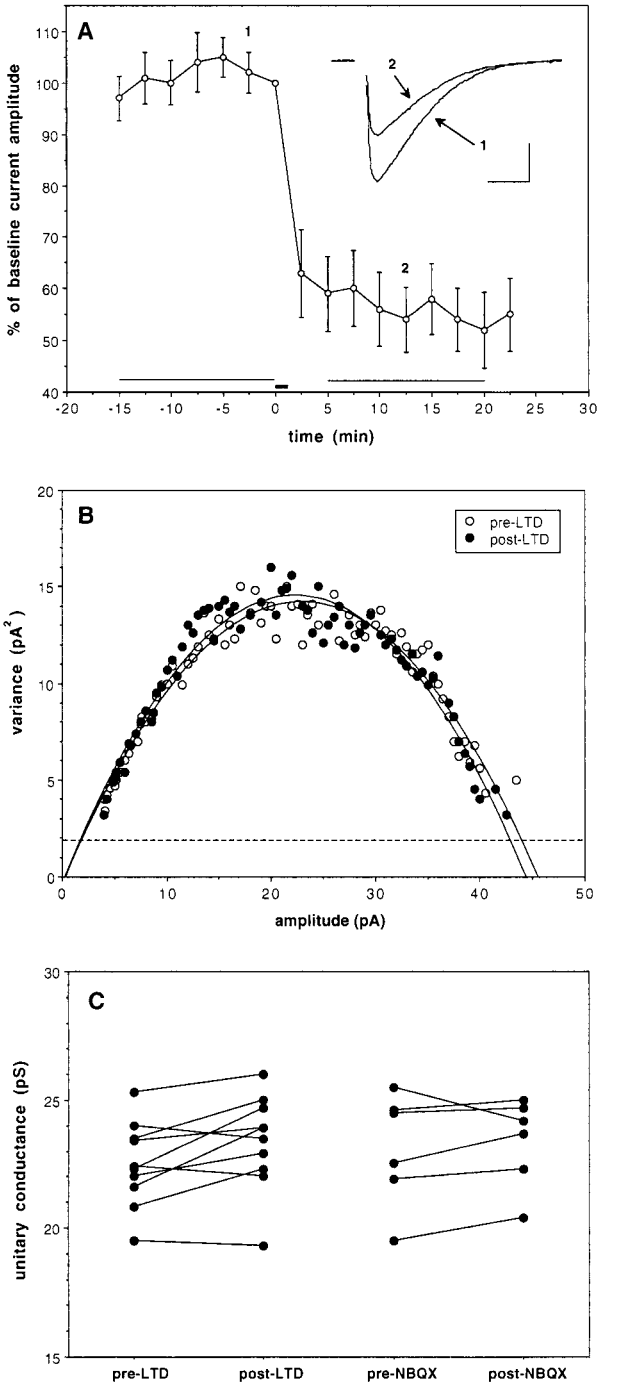

Fig. 5. Peak-scaled nonstationary fluctuation analysis fails to show changes in γ after induction of LTD in cultured granule cell–PC pairs. (*A*) LTD was induced by granule cell-depolarization conjunction (at $t = 0$ min, indicated by the heavy horizontal bar). (*Inset*) Averages of 40 granule cell-evoked EPSCs recorded in a representative PC at the times indicated. The light horizontal lines show the periods in which evoked EPSCs were collected for subsequent peak-scaled nonstationary fluctuation analysis. (Bars = 10 msec, 10 pA.) (*B*) Peak-scaled nonstationary fluctuation analysis: a current–variance relationship for a representative granule cell–PC pair. The variance in the fluctuation of the decays of individual EPSCs recorded either before or after LTD induction is plotted as a function of the mean EPSC-decay amplitude. The dashed line indicates the background variance; the solid lines are parabolic least-squares fits for each group of points. (*C*) Estimates of unitary conductance (γ) based upon parabolic fits for 10 cells in which LTD was induced and 6 cells exposed to a submaximal dose (0.1 μM) of the AMPA-receptor antagonist NBQX. Horizontal lines connect the pre- and postdata points for individual cells.

system and lifted PCs failed to show changes in kinetic properties, including the extent or rate of recovery from desensitization. The negative conclusion regarding AMPA-receptor agonist affinity also was measured directly by constructing dose-response curves to rapidly applied glutamate before and after LTD induction. Finally, the negative conclusion regarding AMPA-receptor unitary conductance was supported by estimates of γ before and after LTD induction produced by peak-scaled nonstationary fluctuation analysis in granule cell–PC pairs.

There are certain caveats that should be sounded in interpreting these data. First, these experiments rely on a cell-culture model system, the properties of which may not be completely preserved in mature, intact tissue. Second, although the experiments that have measured evoked EPSCs and mEPSCs monitored the status of synaptic AMPA receptors, experiments that used application of exogenous glutamate or kainate, either with iontophoretic pulses or in a fast application system, monitored a mixture of synaptic and nonsynaptic AMPA receptors. Whereas the results arguing against AMPA-receptor kinetic changes used both synaptic and exogenous stimulation and the

results arguing against changes in AMPA-receptor unitary conductance used solely synaptic stimulation, the experiment illustrated in Fig. 4 to address AMPA-receptor affinity used solely exogenous stimulation. Thus, there is a formal possibility that changes in the glutamate affinity of synaptic AMPA receptors might have remained below the threshold for detection. However, we believe this possibility to be unlikely, as a previous report found identical glutamate affinity and receptor kinetics when comparing somatic vs. dendritic PC AMPA receptors using outside-out patch recording in a slice preparation (21). Finally, it should be cautioned that those experiments that used perforated-patch recording (Figs. 3 and 4) are likely to overestimate the time course of AMPA-receptor kinetics as a consequence of high R_{series}. However, there is no reason to suspect that this possibility would mask a difference between LTD and control groups.

Some previous work is consistent with the present conclusion that AMPA-receptor kinetics are not changed during LTD expression. In particular, LTD of parallel fiber-PC synapses in slices of young rat induced by conjunctive stimulation of parallel fibers with depolarization (22) or climbing-fiber activation (23) was not found to be associated with changes in the shape of parallel-fiber EPSCs. However, a different conclusion (24) has been reached in experiments that have used aniracetam, a drug that both increases the deactivation time constant and blocks desensitization of PC-AMPA receptors (25). Preincubation of cerebellar slices with aniracetam was found to block significantly induction of cerebellar LTD in a slice preparation (24). Furthermore, the potentiation of EPSC amplitude produced by aniracetam was found to be larger on parallel-fiber synapses that previously had undergone LTD, as compared with control synapses. These experiments were taken to indicate that LTD was expressed, at least in part, as changes in AMPA-receptor kinetics, a position that has been amplified by the construction of a kinetic model that can reproduce aspects of these findings (26). This result is in direct contrast to the present failure to block or occlude LTD induction with cyclothiazide (Fig. 2*A*) or vice-versa (Fig. 2*B*) and may result from differences between the drugs used. For example, it has been proposed that aniracetam modulates desensitization as a consequence of slowing channel closing, whereas cyclothiazide modulates desensitization by stabilizing a nondesensitized agonist-bound closed state (27).

It is worthwhile to note that use-dependent plasticity at other glutamatergic synapses has been suggested to result from several forms of AMPA-receptor modification. In particular, through aniracetam pretreatment experiments similar to those described above, it has been suggested that the expression of LTP at the hippocampal Schaffer collateral-CA1 synapse might involve changes in AMPA-receptor kinetics (28). Hippocampal LTP also has been associated with an increase in AMPA-receptor unitary conductance, as estimated by nonstationary fluctuation analysis (20). An increase in the unitary conductance of GluR1-AMPA receptors expressed in a heterologous system has been observed after phosphorylation of GluR1 Ser-831 by CaMKII (29); this increase may underlie the former observation in hippocampal synapses. However, at present, the main thrust of effort in hippocampal synaptic plasticity has been to investigate regulated postsynaptic AMPA-receptor insertion and internalization as the cellular substrates for hippocampal LTP and LTD, respectively.

In summary, no evidence could be found to link cerebellar LTD expression with alterations in AMPA-receptor kinetics, agonist affinity, or unitary conductance. Thus, at present, the most likely candidate mechanism for cerebellar LTD expression is a reduction of the number of functional AMPA receptors in the postsynaptic membrane, as has been suggested by both electrophysiological (9, 13) and immunocytochemical (10) investigations. These previous observations showed that treatments that block or occlude clathrin-mediated endocytosis produced a near-complete inhibition of LTD. Taken together with the present findings, these results suggest that cerebellar LTD may be expressed solely as a reduction in the number of functional AMPA receptors in the postsynaptic density.

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