Inositol-1,4,5-Trisphosphate Receptor-Mediated Ca Mobilization Is Not Required for Cerebellar Long-Term Depression in Reduced Preparations

KALYANI NARASIMHAN, ISAAC N. PESSAH, AND DAVID J. LINDEN

1Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205; and 2Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California 95616

Narasimhan, Kalyani, Isaac N. Pessah, and David J. Linden. Inositol-1,4,5-trisphosphate receptor-mediated Ca mobilization is not required for cerebellar long-term depression in reduced preparations. J. Neurophysiol. 80: 2963–2974, 1998. Cerebellar long-term depression (LTD) is a cellular model of information storage in which coincident parallel fiber and climbing fiber activation of a Purkinje neuron (PN) gives rise to a sustained attenuation of parallel fiber–PN synaptic strength. Climbing fiber and parallel fiber inputs may be replaced by direct depolarization of the PN and exogenous glutamate pulses, respectively. The parallel fiber–PN synapse has a high-density of mGluR1 receptors that are coupled to phosphoinositide turnover. Several lines of evidence indicated that activation of mGluR1 by parallel fiber stimulation is necessary for the induction of cerebellar LTD. Because phosphoinositide hydrolysis has two initial products, 1,2-diacylglycerol and inositol-1,4,5-trisphosphate (IP3), we wished to determine whether IP3 signaling via IP3 receptors and consequent Ca mobilization were necessary for the induction of cerebellar LTD. First, ratio imaging of free cytosolic Ca was performed on both acutely dissociated and cultured PNs. It was determined that the threshold for glutamate pulses to contribute to LTD induction was below the threshold for producing a Ca transient. Furthermore, the Ca transients produced by depolarization alone and glutamate plus depolarization were not significantly different. Second, the potent and selective IP3 receptor channel blocker xestospongic C was not found to affect the induction of LTD in either acutely dissociated or cultured PNs at a concentration that was sufficient to block mGluR1-evoked Ca mobilization. Third, replacement of mGluR activation by exogenous synthetic diacylglycerol in an LTD induction protocol was successful. Taken together, these results suggest that activation of an IP3 signaling cascade is not required for induction of cerebellar LTD in reduced preparations.

INTRODUCTION

Cerebellar long-term depression (LTD) is a cellular model system of information storage (Ito et al. 1982) that has been proposed to underlie several forms of motor learning, including associative eyelink conditioning, limb load adjustment, and adaptation of the vestibuloocular reflex (Ito 1989; Llisberger 1998; Raymond et al. 1996, Thompson 1986; but see Llinas et al. 1997). Cerebellar LTD is produced when climbing fiber and parallel fiber inputs to a Purkinje neuron (PN) are stimulated conjunctively at low frequencies and is expressed as an attenuation of parallel fiber–PN synaptic strength. In both slice and culture preparations, it has been shown that parallel fiber stimulation may be replaced by exogenous pulses of glutamate, the transmitter of the parallel fibers, and that climbing fiber stimulation may be replaced by direct depolarization of the PN, if it is sufficient to activate voltage-gated Ca channels in the PN dendrite. A series of experiments has shown that climbing fiber activation contributes to LTD induction through Ca influx, whereas glutamate released from the parallel fibers acts on α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and mGluR1 receptors (for review see Linden 1996).

The first evidence indicating that activation of metabotropic receptors was required came from experiments showing that agonists that activated both AMPA and metabotropic receptors (such as glutamate and quisqualate) could substitute for parallel fiber activation during LTD induction but that agonists that failed to activate metabotropic receptors (such as AMPA or aspartate) could not (Kano and Kato 1987; Linden et al. 1991). Complementary evidence was found where metabotropic receptor antagonists blocked LTD induction (Hartell 1994; Lev-Ram et al. 1997; Linden et al. 1991; Narasimhan and Linden 1996). These results, although they indicated that metabotropic receptor activation was required, did not specify which metabotropic receptor(s) were important for LTD induction. The PN expresses a particular metabotropic receptor, mGluR1, at high levels in the dendritic spines where parallel fiber synapses are received (Martin et al. 1992). The first findings to address this issue were those of Shigemoto et al. (1994), who demonstrated that specific inactivating antibodies directed against mGluR1 could block LTD induction in cell culture. This result was confirmed and extended by two different groups with mGluR1 knockout mice (Aiba et al. 1994; Conquet et al. 1994).

Activation of mGluR1 results in the production of two initial products, inositol-1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol. The former binds to specific intracellular IP3 receptors resulting in the liberation of Ca from internal stores, and the latter results in activation of protein kinase C, a process that is also necessary for LTD induction (De Zeeuw et al. 1998; Linden and Connor 1991; see Linden 1996 for review). Given that activation of mGluR1 and

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protein kinase C (PKC) are both required for the induction of cerebellar LTD, it becomes necessary to determine whether the other limb of the mGluR1 signaling pathway mediated by IP3-induced Ca mobilization is also required. PNs express IP3 receptors, particularly the type I isoform, at unusually high levels (Nakanishi et al. 1991), and it has been shown through photolysis of caged IP3 that these receptors are functionally coupled to intracellular Ca release in situ (Khodakhah and Ogden 1993, 1995; Wang and Augustine 1995). Initial attempts to address the role of this signaling cascade in LTD induction were hampered by a lack of specific IP3 receptor antagonists. Application of heparin, a nonspecific (Bezprozvanny et al. 1993; Ehrlich et al. 1994; Herbert and Maffrand 1991) inhibitor of the IP3 receptor (Ghosh et al. 1988), blocked LTD induced by glutamate/depolarization conjunction in cultured PNs (Kasono and Hirano 1995) or by parallel fiber/depolarization conjunction in PNs in a cerebellar slice (Khodakhah and Armstrong 1997). Application of thapsigargin, a drug that depletes internal Ca stores through inhibition of the endoplasmic reticulum Ca-ATPase, was similarly effective (Kohda et al. 1995). However, in slices, it was found to block depression induced by bath application of the mGlur agonist ACPD together with depolarization but not parallel fiber/depolarization conjunction (Hemart et al. 1995). Thapsigargin would be expected to deplete Ca stores gated by both the IP3 receptor and the ryanodine receptor, the latter of which mediates Ca-induced Ca release.

![Image of a cultured Purkinje neuron dendrite with analysis boxes for the dendritic shaft (Sh) and spines (Sp) superimposed.](image-url)
FIG. 2. Glutamate pulses of the duration used in long-term depression (LTD) experiments are subthreshold for evoking Ca transients in cultured PNs. A cultured PN was sequentially stimulated with a series of glutamate pulses of varying duration. The iontophoretic ejection current was held constant. In each case, the Ca levels were allowed to return to baseline between pulses. Measurements were made in the dendritic shaft compartment. Each point represents the mean of the peak Ca level achieved with 4 consecutive stimuli in each of 5 different PNs. Bars represent the SE in this and all subsequent figures. Measurements were made in PNs perfused with internal saline containing either 0.2 or 1 mM fura-2. Note that significant Ca transients were only detected for pulse durations $\lesssim 500$ ms. By comparison, LTD is typically induced with glutamate pulses $\lesssim 110$ ms.

We sought to test the hypothesis that IP3 receptor-mediated Ca mobilization is necessary for cerebellar LTD induction in three ways. First, microfluorimetric Ca measurements were made in both cultured and acutely dissociated Purkinje cells to determine whether the threshold for evoking mGluR-mediated Ca mobilization correlated with the threshold for inducing LTD with exogenous glutamate pulses. Second, a novel, potent and specific IP3 receptor ion channel blocker, xestospongic C (Gafni et al. 1997), was applied to both cultured and acutely dissociated PNs in an attempt to alter LTD induction. Third, activation of mGluRs was replaced in an LTD induction protocol by the application of exogenous synthetic diacylglycerols to determine whether activation of one limb of mGluR1 signaling would be sufficient to cause LTD induction when combined with activation of AMPA receptors and voltage-gated Ca channels.

METHODS

Cell preparation

Embryonic mouse cerebellar cultures were prepared and maintained according to the method of Schilling et al. (1991). Cultures were maintained in vitro for 9–16 days at the time of use in electrophysiological experiments. Acutely dissociated rat PNs were prepared from 7- to 11-day-old animals according to a slight modification of a protocol developed by Mintz and Bean (1993), as

FIG. 3. Cerebellar LTD induced in bis-fura-2 loaded PNs in culture. Whole cell patch-clamp recordings were made from cultured PNs in response to test pulses of glutamate. At $t = -7.5$ min, glutamate pulses were suspended, and 6 3-s-long depolarizing steps to 0 mV were delivered at 0.05 Hz (indicated by horizontal bar marked ‘‘depol’’). Resumption of glutamate pulses indicated that this treatment produced a small, transient depression. Then, at $t = 0$ min, 6 3-s-long depolarizing steps to 0 mV were delivered at 0.05 Hz, each coinciding with a glutamate pulse (indicated by horizontal bar marked ‘‘glu/depol’’), after which glutamate test pulses were resumed, which revealed robust LTD induction. Current traces are from representative single cells at the times indicated on the graph. Scale bars = 2 s, 30 pA. $n = 6$ cells.
previously described (Narasimhan and Linden 1996) with the exception that in some cases the animals were perfused intracardially with ice-cold saline before removal of the cerebellum.

Electrophysiology

Patch electrodes were attached to PN somata and were used to apply a holding potential of −80 mV. Iontophoresis electrodes (1-µm tip diameter) were filled with glutamate, AMPA, or quisqualate (10 mM, in 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), pH 7.1 with NaOH) and were positioned ∼20 µm away from large-caliber dendrites. Test pulses were delivered with negative current pulses (600–800 nA, 30–110-ms duration) applied at a frequency of 0.05 Hz. After acquisition of baseline responses, six conjunctive stimuli were applied, each consisting of a glutamate test pulse combined with a 3-s-long depolarization step to 0 mV, timed so that the depolarization onset preceded the glutamate pulse by 500 ms. Cells were bathed in a solution that contained (in mM) 140 NaCl, 5 KCl, 2 CaCl₂, 0.8 MgCl₂, 10 HEPES, 10 glucose, 0.005 tetrodotoxin (TTX), and 0.1 picrotoxin, adjusted to pH 7.35 with NaOH, which flowed at a rate of 0.5 ml/min. For conventional whole cell recording, the recording electrode contained (in mM) 135 CsCl, 10 HEPES, 1 EGTA, 4 Na₂-ATP, and 0.4 Na-GTP, adjusted to pH 7.35 with CsOH. 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 previously. For perforated-patch recording, electrodes were fabricated as described, with the exception that they were somewhat smaller, yielding a resistance of 4–6 MΩ when filled with the following internal saline (in mM): 95 Cs₂SO₄, 15 CsCl, 8 MgCl₂, and 10 HEPES, pH 7.35 with CsOH. Electrode tips were filled with a small amount of this solution, and the shanks 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. Membrane currents were recorded with an Axopatch 200A amplifier in resistive voltage-clamp mode, low-pass filtered at 5 kHz, and stored on a chart recorder. Access and input resistance were monitored with small hyperpolarizing pulses, and experiments were discarded if either value changed by >20%.

Ca imaging

Fura-2 ratio imaging of intracellular free Ca was accomplished by measuring the background corrected fluorescence ratio at 340 and 380 nm excitation with a cooled charge-coupled device (CCD) camera system. Ca was measured with cells in which fura-2 pentapotassium salt (200 µM) was added to an internal saline consisting of (in mM) 135 CsCl, 10 HEPES, 4 Na₂-ATP, and 0.4 Na-GTP, adjusted to pH 7.35 with CsOH. In one case, a higher concentration of fura-2 was used (1 mM in Fig. 2), and in another bis-fura-2 hexapotassium salt (100 µM) was used in place of fura-2. Light from a 100-W mercury burner was switched by a galvanometer-driven mirror assembly through two optical paths containing 340- and 380-nm excitation filters. (DX-1000 Optical Switch, Solamere Technology Group). The light was then recombined in a liquid light guide coupled to the epifluorescence train of a Zeiss Axiointer 100 with a ×40 1.3NA oil-immersion objective. Emission at 505 nm was passed through a dichroic mirror and focused on the chip (Thompson 7883) of a slow-scan cooled CCD camera (Photometrics CH250). Exposure times were 50–400 ms per single wavelength image. Experiments were conducted at room temperature. A movie of 25 consecutive background-subtracted ratio images was used to capture Ca changes during LTD inducing stimuli (glutamate iontophoresis alone, depolarization alone, or depolarization/glutamate conjunction, as illustrated in Figs. 1–3). Because it was impossible to continuously image all six pulses caused by constraints on storage space, the first and the last pulse were imaged for all conditions. Analysis boxes were constructed for specific regions of interest, and free Ca values were then averaged within the box and plotted versus time. A threshold amplitude for measuring 380 nm-excited signals in each pixel was set at 20 (on the arbitrary scale of the 12-bit camera). This did not result in clipping of peak depolarization-evoked signals as assessed in a limited number of trials with 8 mM external Ca (data not shown).

Reagents

Synthetic diacylglycerols were purchased from Avanti Polar Lipids (Alabaster, AL) and were prepared as 30-mM stock solutions in 100% dimethyl sulfoxide before dilution in external saline and subsequent sonication to disrupt micelles. Xestospongion C was isolated from the marine sponge Xestospongia sp. according to the method of Gafni et al. (1997). It was prepared as a 5-mM stock solution in 100% methanol before dilution in internal saline. (+)-MCPG was obtained from Tocris (Ballwin, MO), and fura-2 was obtained from Molecular Probes (Eugene, OR). All other compounds were from Sigma (St. Louis, MO).

RESULTS

If glutamate contributes to LTD induction through mGluR1-activated, IP3R-mediated Ca mobilization, then it should be possible to measure the Ca transient produced by a glutamate pulse of the type used in LTD induction. To test this hypothesis, cultured PNs were loaded with fura-2 via the patch pipette, and Ca transients evoked by glutamate, depolarization steps, and glutamate/depolarization conjunction were measured in the section of proximal spiny dendrite immediately adjacent to the glutamate iontophoretic pipette (Fig. 1). The glutamate and depolarization pulse parameters were chosen to match those that would be used for LTD induction. In this configuration, our imaging system was capable of resolving laterally projecting spines in 6 of 11 PNs. When glutamate pulses (70-msec duration) were given alone, no significant Ca transient could be detected in either dendritic shaft (peak ΔCa₂ = 3 ± 4 nM, first pulse and 6 ± 5 nM, sixth pulse, mean ± SE, n = 11) or dendritic spine compartments (peak ΔCa₂ = 6 ± 6 nM, first pulse and 5 ± 5 nM, sixth pulse, n = 6). Because spines projecting normal to the plane of focus could not be resolved, measurements from dendritic shaft are likely to have some spine signal contained within them even in those PNs where laterally projecting spines could be resolved. Although our typical image sampling rate of 1 Hz is appropriate for detecting Ca transients evoked by depolarization steps, it is possible that the Ca transients evoked by glutamate pulses are too brief to be detected at this temporal resolution. Although we are limited by the speed of our slow-scan cooled CCD camera, we were able to increase this sampling rate to 4 Hz for a subset of experiments. A sample record shown in Fig. 1D indicates that glutamate-evoked Ca transients were not detected at this higher temporal resolution. Taken together, these measurements indicate that glutamate pulses of the amplitude typically used in an LTD-induction protocol are subthreshold for evoking a Ca transient in a voltage-clamped cultured PN. To examine this point more systematically, the peak amplitude of the glutamate-evoked Ca transient was measured as a function of glutamate pulse duration (Fig. 2). To ensure that we would be able to detect small Ca tran-
transients, measurements were made with two different concentrations of fura-2 in the patch pipette, 200 μM (our standard concentration) and 1 mM, which should be more sensitive. It was found that the smallest pulse duration at which a significant Ca transient was evoked in the dendritic shaft was 500 ms (37 ± 9 nM with 0.2 mM fura-2 and 30 ± 8 nM with 1 mM fura-2, n = 5 cells/group). It should be noted for comparison that spontaneous variations in Ca, of ~10 nM are common, even in PNs bathed in TTX (data not shown).

These results are consistent with a previous report using cultured PNs bathed in Ca-free saline in which large Ca transients (~500 nM) were evoked by 1- to 3-s-long pressure pulses of an mGluR agonist, trans-ACPD (Linden et al. 1994). In contrast, the measurements illustrated in Figs. 1 and 2 were conducted in Ca-containing medium. Although the cell was bathed in TTX and voltage clamped at ~80 mV, it is certainly possible that local voltage-clamp breakdown could have allowed for some Ca entry through voltage-gated channels.

Although glutamate pulses alone do not evoke a Ca transient, it is possible that they synergize with depolarization-evoked Ca transients during LTD induction. To address this possibility, Ca transients evoked by depolarization alone and glutamate/depolarization conjuction were compared. It was observed that the Ca transients evoked by these two modes of stimulation were similar (Fig. 1). When depolarization pulses (3-s duration) were given alone, large Ca transients could be detected in either dendritic shaft (peak ΔCa i = 485 ± 46 nM, first pulse and 466 ± 50 nM, sixth pulse, n = 11) or dendritic spine compartments (peak ΔCa i = 483 ± 61 nM, first pulse and 473 ± 58 nM, sixth pulse, n = 6). When glutamate/depolarization conjunctive stimulation was applied, dendritic shaft (peak ΔCa i = 470 ± 47 nM, first pulse and 459 ± 50 nM, sixth pulse, n = 11) and dendritic spine (peak ΔCa i = 475 ± 62 nM, first pulse and 460 ± 66 nM, sixth pulse, mean ± SE, n = 6) Ca transients of similar amplitude were recorded. Similarly, within-cell comparisons show that the difference between peak Ca transients evoked by glutamate/depolarization conjunctive stimulation and depolarization pulses alone were minimal (dendritic shaft: −10 ± 15 nM, first pulse and −12 ± 18 nM, sixth pulse; dendritic spine: −6 ± 12 nM, first pulse and −9 ± 14 nM, sixth pulse).

Because the K d of fura-2 for Ca is relatively low (~135 nM), it is possible that the fura-2 signal is saturating at the peak of the depolarization response, thereby obscuring the difference between glutamate/depolarization conjunctive stimulation and depolarization pulses alone. To address this possibility, we compared Ca transients in these two conditions with bis-fura-2, a Ca reporter with a lower affinity for Ca (~770 nM) that is also brighter than fura-2 due to the presence of two fluorophores, thus allowing for the use of a lower concentration of dye. The combination of a lower dye concentration and a higher K d for Ca allows for the measurement of Ca concentration and the reliable induction of LTD of glutamate-evoked conjunctive responses in the same cells (Fig. 3). In bis-fura-2-loaded cells (100 μM in pipette solution), after acquisition of a brief set of baseline responses to glutamate test pulses, depolarization alone produced no significant alteration, whereas subsequent glutamate/depolarization conjunctive stimulation induced LTD (56 ± 8% of baseline at t = 40 min, n = 6). In these same cells, no significant differences were seen between glutamate/depolarization conjunctive stimulation (dendritic shaft: peak ΔCa i = 536 ± 53 nM, first pulse, n = 6; dendritic spine: 549 ± 68 nM, n = 3) and depolarization pulses alone (dendritic shaft: 558 ± 58 nM, n = 6; dendritic spine: 526 ± 70 nM, n = 3). Similar results were seen when the sixth pulse was analyzed (data not shown). Thus it appears as if glutamate pulses typically used in LTD induction protocols do not contribute to the associated Ca transients in cultured PNs.

As an alternative means of addressing this issue, measurements of Ca transients evoked by LTD-inducing glutamate/depolarization conjunctive stimulation and its component parts were repeated with an acutely dissociated PN preparation that lacks spine compartments but that expresses LTD in a manner that so far was seen to be indistinguishable from cultured PNs (Narasimhan and Linden 1996). Similar results are seen with both preparations (Fig. 4). Glutamate pulses alone do not evoke significant Ca transients, and the transients evoked by conjunctive stimulation are not larger than those evoked by depolarization alone. One disadvantage of the acutely dissociated PN preparation is that the Ca homeostatic capacity of the cell is sometimes compromised by the dissociation procedure. Thus it may be seen that the basal Ca concentration is somewhat elevated in the later trials (depot 6, conj 1, conj 6). Because the experiments shown in Figs. 1 and 4, A–C, were always conducted in a manner in which depolarization preceded glutamate/depolarization conjunctive stimulation, there is the possibility that conjunctive stimuli might be larger if they were delivered first. Figure 4D illustrates an experiment in which this was performed. Conjunctive stimuli were not larger even when they were the first manipulation, making it unlikely that the depletion of an internal Ca store or some other form of run-down underlies a failure to detect larger conjunction responses. A comparison of the depolarization and conjunctive Ca responses may be expressed as a ratio of their amplitudes. Figure 5 shows these values for the complete population of acutely dissociated PNs tested. Although the range of ratios was large, the means were >1 in every measure of the Ca transients (first pulse peak amplitude = 1.26 ± 0.12, first pulse area = 1.22 ± 0.29, sixth pulse peak amplitude = 1.13 ± 0.08, sixth pulse area = 1.13 ± 0.11, n = 7), indicating that there is no systematic increase of the Ca transient with glutamate/depolarization conjunctive stimulation compared with depolarization alone. To address the possibility that our failure to detect such an increase results from near-maximal stimulation of depolarization-evoked Ca transients, we performed an analysis relating the area of the depolarization-evoked Ca transient to the depolarization/conjunctive area ratio. If the Ca transient evoked by depolarization alone were sometimes large enough to occlude an additive or synergistic effect of depolarization/glutamate conjunction, one would expect to find a significant negative correlation between these two measures. However, with the two-tailed Pearson r test, no significant correlation was seen for either the first (r = 0.29, P > 0.20, n = 7) or the sixth (r = 0.19, P > 0.20, n = 7) pulse.

If IP3 receptor-mediated Ca mobilization was necessary for induction of cerebellar LTD, application of drugs that
interfere with the former would be expected to block the latter. First, we screened some inhibitors of IP3 signaling to confirm their effectiveness in blocking mGluR-evoked IP3-mediated Ca mobilization in cultured PNs (Fig. 6A). This was done by assessing fura-2 Ca signals resulting from application of the mGluR agonist quisqualate in Ca-free external saline (control: $\Delta C_{ai} = 158 \pm 22 \text{nM}, n = 10$). With internal application, both the novel IP3 receptor ion channel blocker, xestospongin C (1 $\mu$M) and the nonspecific polyamine heparin (100 or 500 $\mu$g/ml) produced a nearly complete blockade of Ca mobilization ($\Delta C_{ai} = 12 \pm 3 \text{nM}, 17 \pm 4 \text{nM},$ and $13 \pm 2 \text{nM},$ respectively, $n = 10$ cells/group). The mGluR antagonist (+)-MCPG (500 $\mu$M) was similarly effective ($\Delta C_{ai} = 12 \pm 4 \text{nM}, n = 10$). Because LTD induction requires Ca influx through voltage-gated channels, it became necessary to determine whether any of these compounds also affected Ca influx. This was measured by applying a voltage step to a PN identical to that used in the LTD induction protocol (0 mV, 3 s) in normal (2 mM Ca) external saline. None of the compounds tested produced a significant alteration in depolarization-evoked Ca influx (control: $\Delta C_{ai} = 429 \pm 45 \text{nM}, n = 10$), although heparin at 500 $\mu$g/ml produced a small attenuation that might become significant if $n$ were increased ($\Delta C_{ai} = 344 \pm 39 \text{nM}, n = 10$). This same set of compounds was then assessed for its effects on LTD induction produced by glutamate depolarization conjunction (Fig. 6B). Xestospongin C produced no alteration in the amplitude or time course of LTD when applied at a concentration (1 $\mu$M) previously shown to produce a nearly complete block of IP3 receptor-mediated Ca mobilization ($57 \pm 8\%$ of baseline at $t = 40 \text{ min}, n = 5$). A similar negative result with xestospongin C was observed.
with the same LTD induction protocol in acutely dissociated PNs (57 ± 13% of baseline at t = 20 min, n = 3). In contrast, the nonspecific polyanion heparin produced a partial dose-dependent blockade of LTD amplitude (65 ± 7 and 75 ± 7% of baseline at t = 40 min for 100 and 500 μg/ml respectively, n = 5 cells/group). It should be noted that the attenuation of LTD amplitude produced by heparin is unlikely to be related to its blockade of IP3 receptors as these doses both produce a similar and nearly complete blockade of IP3 receptor function, and yet the lower dose fails to produce a significant inhibition of LTD. (+)-MCPG (500 μM), an antagonist of mGluRs, blocked LTD induction as previously reported in other preparations (Hartell 1994; Lev-Ram et al. 1994) but IP3 receptor activation were not, then it seems likely that 1,2-diacylglycerol would be the relevant signaling molecule. To test this hypothesis, a series of synthetic 1,2-diacylglycerols was applied to cultured PNs and the responses of these PNs to exogenous pulses of AMPA was monitored by perforated patch recording (Fig. 7). Application of diacylglycerols while AMPA test pulses were continued produced no significant alteration in the test pulses’ response. However, when the unsaturated diacylglycerols oleoylactetylglycerol (10 μM) and dioleoylglycerol (10 μM) were applied together with depolarizing steps and AMPA test pulses, a slowly developing depression was seen (45 ± 6 and 46 ± 8% of baseline, respectively, at t = 45 min, n = 6 cells/group). A saturated diacylglycerol, dipalmitylglycerol (10 or 30 μM), produced no sustained depression when paired with depolarizing pulses (101 ± 8 or 100 ± 9% of baseline, respectively at t = 45 min, n = 6).

Given that the depressions produced by quisqualate/depolarization conjunction and unsaturated diacylglycerols plus depolarization develop with such different time courses, it is possible that they represent two forms of LTD that are differently expressed? Although we have no direct evidence to explain the slow time course of the latter depression, it is likely to result from the kinetics of membrane partitioning and degradation of diacylglycerol. A similar slowly developing depression is produced in PNs by the bath application of PKC-activating phorbol esters (Linden 1994; Linden and Connor 1991). More importantly, several lines of direct evidence suggest that the application of unsaturated diacylglycerols is effectively substituting for mGluR1 activation in LTD induction. First, subsequent presentation of quisqualate/depolarization conjunction produced LTD of the PNs previously treated with dipalmitylglycerol (67 ± 8% of baseline at t = 60 min) but produced no further alteration of the depressed responses in the cells treated with oleoylactetylglycerol (44 ± 5% of baseline at t = 60 min) or dioleoylglycerol (46 ± 8% of baseline at t = 60 min). Second, the pharmacological profile of LTD produced by unsaturated diacylglycerol/depolarization conjunction is consistent with an action of the diacylglycerol downstream from mGluR1 but upstream from PKC (Fig. 8). In a separate set of experiments using conventional whole cell recording, addition of the IP3 receptor antagonist xestospongin C (1 μM) to the internal saline or the mGluR antagonist (+)-MCPG (500 μM) to the external saline failed to block the attenuation produced by oleoylactetylglycerol (10 μM) plus depolarization. In contrast, addition of the PKC inhibitor chelerythrine (10 μM) to the internal saline completely suppressed the development of persistent depression seen with this protocol.

**DISCUSSION**

The main conclusion of this set of experiments is that, in the current reduced preparations, activation of a postsynaptic mGluR1/IP3 cascade by glutamate does not produce a Ca signal that is required for induction of cerebellar LTD, either...
FIG. 6. Xestospongin C, a specific and potent IP3 receptor ion channel blocker, fails to alter LTD induction. A: peak Ca transients were measured in the portion of proximal dendritic shaft that was immediately adjacent to the quisqualate-containing pipette in fura-2-loaded PNs grown in culture. Ca transients evoked by a 3-s depolarization to 0 mV in normal (2 mM) external Ca were measured as an index of voltage-gated Ca channel function (labeled as “depol evoked”). Ca transients evoked by a pulse of 100 μM quisqualate in 0 Ca/0.2 mM EGTA (free Ca ~20 nM) external saline (6 psi, 2 s) were measured as an index of mGluR1 function (labeled as “quis evoked”). The IP3 receptor blockers xestospongin C and heparin were added to the internal saline and were perfused via the patch pipette for 15 min before imaging. The metabotropic receptor antagonist (−)-MCPG was applied in the bath 20 min before Ca measurements; n = 6 cells/group for depolarization-evoked Ca influx and n = 10 cells/group for quisqualate-evoked Ca mobilization. B: cerebellar LTD recorded in whole cell voltage-clamp mode in cultured PNs was monitored with glutamate test pulses and was evoked by glutamate/depolarization conjunction (indicated by horizontal bar at t = 0 min). All compounds were applied in the internal saline with the exception of (−)-MCPG, which was applied in the bath. Current traces are from representative single cells at the times indicated on the graph. Scale bars = 2 s, 50 pA. n = 5 cells/group.

by itself or in a synergistic manner with depolarization. Although application of large glutamate pulses can produce IP3 receptor-mediated Ca mobilization in PNs, the minimum pulse duration necessary to resolve this signal is well above that required for LTD induction. The effector of mGluR1 activation that is necessary for LTD induction in these reduced preparations appears to be 1,2-diacylglycerol (and/or its metabolites).

These results show that, in both spine-bearing cultured PNs and spineless acutely dissociated PNs, large Ca transients were detected by depolarization but that glutamate pulses neither produced detectable Ca transients (at the durations used in LTD experiments) nor did they augment Ca transients when applied together with depolarization. There are two important caveats that should be applied to this finding. First, it is possible that Ca transients could be produced by brief glutamate pulses but that these signals are so spatially restricted that they could not be detected with the present imaging system. If, for example, a Ca-sensitive enzyme were localized immediately adjacent to the mouth of the IP3R ion channel, it is possible that Ca release could activate this enzyme yet remain undetected by imaging. Indeed, this type of signal is likely to remain undetected by the even the most sophisticated multiphoton imaging currently available. Second, both the embryonic cultured PN and the acutely dissociated PN are preparations that have important differences from PNs in situ and consequently could potentially have different Ca signaling properties.

Synaptically evoked Ca signals were extensively studied in PNs with the brain slice preparation (for review see Eilers...
FIG. 7. Exogenous unsaturated diacylglycerols applied together with depolarization produce an LTD-like effect. Perforated-patch recordings were made from cultured PNs in response to test pulses of AMPA. At $t = 0-5$ min, exogenous diacylglycerol (DAG) was bath applied, and AMPA test pulses were continued at 0.05 Hz. At $t = 20-25$ min, the same exogenous diacylglycerol was bath applied together with 10 3-s-long depolarizing steps to 0 mV delivered at 0.05 Hz each coinciding with an AMPA pulse, and at $t = 50$ min quisqualate pulses were applied to the same site on the dendrite as the AMPA test pulses, together with the same depolarizing stimulus, after which AMPA test pulses were resumed. Current traces are from representative single cells at the times indicated on the graph. Scale bars = 2 s, 40 pA. $n = 6$ cells/group.

et al. 1996). Activation of the climbing fiber input to the PN results in a large all-or-none excitatory postsynaptic potential (EPSP), which is followed by a burst of action potentials. This EPSP and its sequela may be completely suppressed by an AMPA receptor antagonist. Imaging experiments have shown that activation of the climbing fiber input is associated with Ca spike firing, which gives rise to a Ca transient that is broadly distributed across the dendritic arbor (Konnerth et al. 1992; Miyakawa et al. 1992; Ross and Werman 1987). The contribution of other processes to climbing fiber-evoked Ca signals, such as Ca-induced Ca release, is unclear. It was reported that climbing fiber-evoked Ca signals are unaffected by an antagonist of ryanodine receptors, ruthenium red, and that they display a monotonic rise, without a secondary component indicating Ca release (Eilers et al. 1995b, 1996; Kano et al. 1995). Nevertheless, repetitive voltage-clamp depolarizations in PNs were reported to cause a supralinear increase in Ca, a portion of which can be blocked by ruthenium red (Llano et al. 1994). A similar result may be seen in cultured PNs, where a different inhibitor of ryanodine receptor signaling, 8-NH$_2$-cADP-ribose, blocked a portion of the depolarization-evoked Ca transient (Linden et al. 1995). Thus it is likely that a portion of the depolarization-evoked Ca transients measured may be attributed to Ca-induced Ca release. The recent demonstration of physical and functional interactions between L-type voltage gated ion channels and ryanodine receptors (Chavis et al. 1996; Nakai et al. 1996) supports this hypothesis.

In contrast to climbing fiber stimulation, parallel fiber stimulation results in a rather localized rise in dendritic Ca.
Low intensity stimulation of parallel fibers evokes graded EPSPs, whereas at higher intensities the EPSPs become suprathreshold and develop a regenerating spike. Recent studies with confocal or two-photon imaging have shown that even subthreshold activation of parallel fibers produces a highly localized rise in postsynaptic Ca that was confined to a small number of spines and the adjacent dendritic branchlet (Denk et al. 1995; Eilers et al. 1995a). The source of these Ca transients appears to be predominantly influx via voltage-gated channels (Eilers et al. 1995a). However, Denk et al. (1995) found that a subpopulation of spines responded with an increase in Ca with hyperpolarizing commands, which is consistent with Ca entry through ligand-gated rather than voltage-gated channels. It is not clear which ligand-gated channel could be the source for Ca influx at this synapse as NMDA receptors are not expressed in mature PNs, and the type of AMPA receptors expressed in PNs are GluR2 containing and thus very weakly permeant to Ca (Linden et al. 1993; Tempia et al. 1996).

Although it is well established that application of exogenous glutamate or mGluR agonists can produce Ca mobilization in PNs in slice (Llano et al. 1991, Vranesic et al. 1991) or culture (Linden et al. 1994), it is unclear whether parallel fiber stimulation is capable of engaging this signal. Although parallel fiber-evoked Ca transients were completely blocked with an antagonist of AMPA receptors, MCPG, an antagonist of mGluR receptors had no effect on these Ca transients (Eilers et al. 1995a). A similar result was obtained with bursts of stimuli applied to the parallel fibers (6 pulses at 50 Hz). This protocol, which elicits a slow MCPG-sensitive EPSP, was not associated with a Ca transient (Batchelor et al. 1996). These results are consistent with the current conclusion that mGluR-evoked IP3-mediated Ca transients are not required for cerebellar LTD induction. It should be noted, however, that this finding may not be generalized to other glutamatergic synapses as synaptically driven mGluR-mediated Ca mobilization signals were detected in hippocampal CA3 pyramidal neurons after high-frequency (50 Hz, 1 s) stimulation (Pozzo Miller et al. 1996).

Previous reports have shown that injection of the IP3R antagonist heparin can block LTD in either cultured PNs (Kasono and Hirano 1995) or PNs in slice (Khodakhah and Armstrong 1997). The concentrations of heparin applied in the patch pipette saline in these studies were 2.5 mg/ml and 50 μg/ml, respectively. Unfortunately, heparin, a polyanionic polymer, is notoriously nonspecific. Two of its side effects that are potentially relevant to LTD induction include inhibition of PKC activity and activation of ryanodine receptors. Heparin inhibits PKC in vitro with an IC50 of 2.8 μg/ml (Herbert and Maffrand 1991). Thus it is likely that the attenuation of LTD seen by Kasono and Hirano (1995) and Kohdakah and Armstrong (1997) as well as that in the present report is related to inhibition of PKC, a process that has previously been shown to be necessary for LTD induction (Linden and Connor 1991; for review see Linden 1996). In addition, heparin has been shown to induce Ca release through ryanodine receptors in skeletal muscle sarcoplasmic reticulum at concentrations of 0.1–1 mg/ml (Bezprozvanny et al. 1993; Ehrlich et al. 1994). It is possible that heparin could function to deplete ryanodine receptor-gated internal stores in PNs as PNs express relatively high levels of the skeletal muscle type ryanodine receptor RyR1. This idea is consistent with a report that three different manipulations that interfere with ryanodine receptor Ca signaling (ruthenium red and ryanodine, which are receptor antagonists, and thapsigargin, a compound that depletes internal stores of Ca through inhibition of the endoplasmic reticulum Ca-ATPase) block LTD induction by glutamate depolarization in culture (Kohda et al. 1995).

In addition to heparin, we made use of the newly described IP3R ion channel blocker, xestospongin C, a marine natural product that has no effects on either voltage-gated Ca influx or ryanodine receptor-mediated Ca signals at the concentrations utilized herein (Gafni et al. 1997) (Fig. 6). The finding
that application of xestospongin C at a concentration that completely blocked mGluR-evoked Ca mobilization was ineffective in altering LTD induction argues against a requirement for this signaling system. As a further test of this idea, synthetic diacylglycerols were applied to PNs and were found to produce an LTD-like effect if combined with depolarization (Figs. 7 and 8). This effect was produced by two different diacylglycerols that contain at least one unsaturated acyl group, in confirmation of a previous report (Linden 1994), but was not produced by a diacylglycerol with two saturated acyl groups. The requirement for an unsaturated acyl group could reflect a requirement for local membrane association or fluidization or possibly a structural requirement for a downstream metabolite of diacylglycerol that is required for LTD induction. This could involve the production of free unsaturated fatty acids (Linden 1995) via the action of diacylglycerol lipase.

However, the current findings do not rule out the possibility that, although IP3 signaling is not absolutely required, it still may contribute to LTD induction and that the degree of this contribution could be different in PNs in situ. The nature of PKC activation is such that unsaturated diacylglycerols reduce the Ca requirement for PKC activation. Thus it is possible that, in the presence of high concentrations of IP3, PKC activation and LTD induction could proceed without a diacylglycerol signal. Likewise, in the presence of higher concentrations of diacylglycerol (as in Fig. 7 and 8), it is possible that IP3/Ca mobilization-independent PKC activation may ensue. This property of PKC could potentially explain two results, which seems to be at odds with those presented here. Khodakhah and Armstrong (1997) have shown that photolysis of IP3 can induce LTD in a slice preparation when combined with depolarization. More recently, Inoue et al. (1998) have shown that LTD induction is blocked in cerebellar slices from type I IP3 receptor knockout mice or in normal cerebellar slices treated with an inactivating antibody directed against this receptor.

One of the most interesting properties of cerebellar LTD is the associative nature of its induction; when reasonable parallel fiber stimulation intensities are used, both parallel fibers and climbing fibers must be activated within a limited temporal window for LTD induction to be successful (for review see Linden 1996). Given what is known about the candidate signaling systems involved in LTD induction, several models may be constructed to explain the associative property in molecular terms. A “calcium threshold” model states that there is a critical Ca concentration for the induction of LTD that is not achieved by either stimulation of parallel fibers alone or climbing fibers alone but which is achieved when both are stimulated together. The detector of the Ca threshold could be the IP3 receptor itself, as its activation is Ca sensitive (Berridge 1993), or a Ca-sensitive enzyme such as PKC. Alternatively, signals of different types, such as the Ca transient evoked by climbing fiber activation (or depolarization) and the diacylglycerol liberation evoked by parallel fiber stimulation (or glutamate), could converge to form the associative trigger. Again, PKC is a good candidate for an associative detector. Formally, these models are not mutually exclusive; there is no reason an associative learning rule could not be built from multiple converging signals at the molecular level. However, the weight of the experimental evidence is against a Ca threshold model, at least in reduced preparations.

We thank T. F. Molinski for analysis of xestospongin C purity by NMR, D. Gurfel for skillful technical assistance, and C. Aizenman, J. Baraban, C. Hansel, R. Hugunin, P. Manis, A. Parent, and K Takahashi for useful suggestions.

This research was supported in part by Public Health Service Grant R01 MH51106, the Develbiss Fund, a McKnight Scholarship, and a Young Investigator Award from the National Alliance for Research on Schizophrenia and Depression to D. J. Linden and Public Health Service Grants RO1 ESO5002 and PO1 AR44750. J. N. Pessah.

Address for reprint requests: D. J. Linden, Dept. of Neuroscience, Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.

Received 6 April 1998; accepted in final form 18 August 1998.

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