An Evaluation of the Nitric Oxide/cGMP/cGMP-Dependent Protein Kinase Cascade in the Induction of Cerebellar Long-Term Depression In Culture

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Cerebellar long-term depression (LTD) is a model system of information storage in which a persistent attenuation of the parallel fiber–Purkinje neuron (PN) synapse is induced by conjunctive stimulation of parallel fiber and climbing fiber inputs at low frequency. As some studies have suggested that release of the gaseous second messenger, nitric oxide (NO), in the molecular layer and the consequent activation of soluble guanylate cyclase and cGMP-dependent protein kinase (PKG) in the PN, is necessary for LTD induction, we have further examined this hypothesis using a cell culture protocol. In cerebellar cultures made from transgenic mice in which the gene for neuronal nitric oxide synthase (nNOS) has been rendered null, LTD induced by glutamate/depolarization conjunctive stimulation was indistinguishable from that in cultures from wild-type mice in terms of amplitude, rate of onset, and duration. Bath application of cGMP analogs produced a large (80%), transient attenuation of glutamate-gated inward currents. However, application of an activator of soluble guanylate cyclase or an inhibitor of type V cGMP-phosphodiesterase did not mimic the effect of cGMP analogs, and inclusion of cGMP analogs in the patch pipette did not give rise to a slowly developing attenuation, suggesting that these compounds exert their effects at the cell surface. Free Ca was measured in the distal dendritic arbor of single PNs by fura-2 microfluorimetry. Cyclic ADP-ribose (cADPr), which has been suggested to be formed by a cGMP/PKG-dependent process and to be an endogenous ligand of the type 2 ryanodine receptor, increased the response to a 3 sec depolarizing step from -80 to $+10$ mV when included in the patch pipette. This effect was completely antagonized by the co-inclusion of a competitive inhibitor (8-NH₂-cADPr). Induction of LTD was not blocked by inclusion in

the patch pipette of three different PKG inhibitors or 8-NH₂-cADPr. These results suggest that while cGMP/cADPr signaling may be important in neuronal Ca regulation, a NO/cGMP cascade is not required for cerebellar LTD induction in culture.

[Key words: long-term depression, cerebellum, nitric oxide, cGMP, cGMP-dependent protein kinase, cyclic-ADP-ribose, Purkinje neuron]

Long-term use-dependent alterations in synaptic communication are thought to underlie information storage in the brain. Experimentally, these synaptic alterations are studied as the phenomena called long-term potentiation (LTP) and long-term depression (LTD). LTP and LTD are blanket terms, each of which refers to several different processes engaged by different patterns of activity and which have varying consequences for the transmission of neuronal signals (e.g., homosynaptic LTP, associative LTP, homosynaptic LTD, heterosynaptic LTD, etc.) At a number of synapses in different brain regions, two or more of these processes can co-occur. One particular form of LTD, which has been demonstrated in the cerebellum, is a persistent, input-specific attenuation of parallel fiber–PN synapses, which results from coactivation of parallel fiber and climbing fiber inputs to a PN at low frequency for several minutes (Ito et al., 1982). Cerebellar LTD is generally understood to have three initial requirements for induction, activation of AMPA receptors, activation of mGluR1 receptors (both of which are produced by glutamate release from parallel fibers) and Ca influx through voltage-gated channels, which is triggered by the climbing fiber EPSP. The activation of mGluR1 receptors results in phospholipase C-mediated production of diacylglycerol and inositol-1,4,5-trisphosphate. AMPA receptor activation exerts its effects by gating Na influx, which might possibly serve to slow Ca extrusion via a Na/Ca exchanger. Some, or possibly all, of these signaling pathways appear to converge to produce activation of the enzyme protein kinase C (PKC), which is necessary for LTD induction. Cerebellar LTD is expressed postsynaptically as a depression of AMPA sensitivity and, at present, has not been found to have a presynaptic component (see Linden, 1994b, for review).

Recently, several studies have suggested that release of the gaseous messenger molecule nitric oxide (NO) and the consequent production of cGMP by soluble guanylate cyclase are necessary for cerebellar LTD induction. Bath application of either NOS inhibitors or an extracellular NO scavenger (hemoglobin) was shown to block LTD induction by parallel fiber/climbing

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fiber conjunctive stimulation (Shibuki and Okada, 1991) or parallel fiber/depolarization conjunctive stimulation (Crepel and Jaillard, 1990; Daniel et al., 1993). Likewise, an LTD-like phenomenon could be produced when climbing fiber stimulation was replaced by bath application of NO donor molecules or membrane permeable analogs of cGMP (Shibuki and Okada, 1991; Daniel et al., 1993). Interestingly, application of cGMP or NO donors directly to the Purkinje neuron (via a patch pipette) also resulted in depression of parallel fiber responses (Daniel et al., 1993; Hartell, 1994), while postsynaptic application of an NO synthase inhibitor did not block LTD induction (Daniel et al., 1993). In contrast, Glaum et al. (1992) found no effect of a bath-applied NO donor on parallel fiber-evoked EPSPs recorded in PN dendrites in a similar acute slice preparation. In cultured PN dendrites, LTD of glutamate currents was unaffected by NOS inhibitors, hemoglobin, or an NO donor (Linden and Connor, 1992).

Assigning a role in LTD induction to NO signaling is complicated by several biochemical observations. While both nNOS and endothelial NOS (eNOS) immunoreactivity and activity are found in granule cells and their parallel fibers and in basket cells, they are absent in PN dendrites (Bredt et al., 1990; Vincent and Kimura, 1992; Dinerman et al., 1994). The presence of NOS in the cells of the inferior olive and their axons, the climbing fibers, is controversial, with some groups finding none (Bredt et al., 1990; Vincent and Kimura, 1992; Ikeda et al., 1993; Chen and Aston-Jones, 1994) and another finding a small amount (Southam and Garthwaite, 1993). Although PN dendrites contain guanylate cyclase (Zwiler et al., 1981; Matsuoka et al., 1992) and cGMP-dependent protein kinase (De Camilli et al., 1984), and have detectable resting levels of cGMP (Sakaue et al., 1988), they do not significantly accumulate additional cGMP in response to application of a NO donor (de Vente et al., 1990; Southam et al., 1992). Together with the observation that LTD may be induced when climbing fiber activation is replaced by direct depolarization of the PN, these findings make it difficult to design a mechanism by which climbing fiber activation triggers NO release, and NO once released acts on PN dendrites.

Several models have been proposed to explain NO/cGMP action in cerebellar LTD. In one, climbing fiber-evoked Ca influx into PN dendrites causes K⁺-efflux, which depolarizes adjacent parallel fiber terminals, resulting in Ca influx and the consequent activation of NOS in this compartment (Crepel et al., 1994). The NO produced by this stimulation activates soluble guanylate cyclase in the PN, and the resultant cGMP activates PKG which, in turn, phosphorylates proteins that contribute to attenuation of AMPA receptor function. Specifically, PKG is thought to phosphorylate G-substrate, resulting in a decrease in the activity of a phosphatase that acts upon AMPA receptors that have been previously phosphorylated by PKC (Ito and Karachot, 1992; Crepel et al., 1994). Thus, in this model, LTD induction requires the concerted activation of PKC and inhibition of a phosphatase (via PKG) to produce AMPA receptors that are suitably phosphorylated and, thereby, functionally attenuated. In a second model, NO produced as a consequence of climbing fiber activation (this model does not specify exactly how this would be accomplished) activates guanylate cyclase/cGMP/PKG in the PN dendrites, resulting in the activation of ADP-ribosyl cyclase and the production of cADPr (Berridge, 1993). cADPr has been proposed to act at the type 2 ryanodine receptor to stimulate its function of Ca-induced Ca release (Galione et al., 1993). The Ca released in this manner is then proposed to diffuse into dendritic spines, where it exerts its effect by potentiating the activation of IP₃-gated Ca stores (Berridge, 1993). Finally, a third model proposes that there is a tonic diffusible signal originating from Bergmann glia that suppresses the induction of LTD and that this signal is blocked (in a manner that is not specified) by NO formed in the PN as a consequence of climbing fiber activation (Shibuki, 1993). The present study is designed to further evaluate the first two of these models.

Materials and Methods

Materials and Methods

nNOS null mice were obtained from a breeding colony established at Johns Hopkins University with animals produced by homologous recombination in the laboratory of M. C. Fishman (Huang et al., 1993). Both male and female mice were screened for homozygosity in the nNOS null mutation by Southern blot analysis as described (Huang et al., 1993) prior to breeding. Mouse embryonic cerebellar cultures were prepared according to the protocol of Schilling et al. (1991) and were maintained *in vitro* for 12–17 d. Whole-cell patch-clamp recording from PN dendrites was done essentially as previously described (Linden and Connor, 1991). Briefly, patch electrodes were attached to PN somata and were used to apply a holding potential of -80 mV. Iontophoresis electrodes (1 μ m tip diameter) were filled with 10 mM glutamate (in 10 mM HEPES, pH 7.2 with NaOH) and were positioned ~ 20 μ m away from large-caliber dendrites. Test pulses of glutamate were delivered with negative current pulses (850 nA, 30 to 110 msec duration) applied at a frequency of 0.05 Hz. Previous work has shown that currents evoked in this recording configuration are mediated almost entirely by AMPA type receptors as assessed by CNQX blockade (Linden and Connor, 1991; Linden et al., 1991). Cells were bathed in a solution that contained NaCl (140 mM), KCl (5 mM), CaCl₂ (2 mM), MgCl₂ (0.8 mM), HEPES (10 mM), glucose (10 mM), tetrodotoxin (0.0005 mM), and picrotoxin (0.1 mM), adjusted to pH 7.35 with NaOH. The recording electrode contained KCl (140 mM), HEPES (10 mM), and EGTA (1 mM) adjusted to pH 7.35 with KOH, except for experiments with combined patch-clamp and fura-2 imaging in which case EGTA was omitted and 150 μ M fura-2, K₂-salt (Molecular Probes, Eugene, OR) was added. Patch electrodes were pulled from N51A glass and polished to yield a resistance of 3–6 M Ω . Membrane currents evoked by glutamate were recorded with an Axopatch 200A amplifier in resistive voltage-clamp mode and stored on a chart recorder that imposed a 0 to 30 Hz bandpass. Experiments were conducted at ambient room temperature.

Imaging of intracellular free Ca was accomplished by measuring the background-corrected fluorescence ratio at 337 and 384 nm excitation using a cooled CCD camera system. Illumination at 337 nm was provided by a pulsed nitrogen laser operating at 20 Hz, and 384 nm was provided by a separate nitrogen-pumped laser using the dye Exalite 384 (Laser Science Inc). This light was passed through a quartz fiber optic Y-conduit to the epifluorescence train of a Zeiss Axiovert 100 equipped with a 40 $\times$ 1.3 NA oil-immersion objective. Emission at 505 nm was passed through a dichroic mirror and was focused on the chip (Thompson 7883) of a slow-scan cooled CCD camera (Photometrics CH250). Exposure times were 200 msec per single wavelength image. Digitized images were acquired to magneto-optical disk using custom software (kindly provided by Dr. D. W. Tank) running on a Macintosh IIfx microcomputer. Free calcium concentration was derived from the F337/F384 ratio measurements using the standard relationship outlined by Grynkiewicz et al. (1985).

Protoporphyrin-IX free acid (Porphyrin Products, Logan, UT), KT5823 (Calbiochem, La Jolla, CA), and chelerythrine (LC Labs, Woburn, MA) stocks were prepared in 100% DMSO prior to dilution in external saline. Protoporphyrin-IX was used in the dark to prevent photoactivation. 8-CPT-cGMP, Sp-cGMP-S, cGMP, Rp-8CPT-cGMP-S (BioLog, La Jolla, CA), RKRARKE peptide (Peninsula Labs, Belmont, CA), and zaprinast (Sigma, St. Louis, MO) were prepared as stocks in ddH₂O. cADPr and 8-NH₂-cADPr were a generous gift of Dr. T. Walseth and were synthesized according to his published method (Walseth and Lee, 1993). All other reagents were from Sigma.

For nNOS immunocytochemistry, cells were washed with TBS (50 mM Tris-HCl, pH 7.4, 1.5% NaCl) and fixed for 30–60 min at 4°C in 4% paraformaldehyde in a 0.1 M phosphate buffer. The cultures were then washed with TBS, followed by permeabilization with 0.2% Triton X-100 in TBS for 5 min. Cultures were then blocked with 4% normal goat serum, 0.1% Triton X-100 in TBS for 60 min, then they were incubated with affinity-purified anti-nNOS antibodies overnight at 4°C.

Figure 1. Cerebellar LTD induced by glutamate/depolarization conjunctive stimulation is not altered in cultured PNIs from nNOS null transgenic mice. Hoffman modulation photomicrographs of nNOS immunocytochemistry in wild-type (*a* and *b*) and nNOS null (*c* and *d*) cerebellar cultures. Small round cells stained in *a* and *b* are likely to be granule cells. No PNIs (as identified by somatic shape and size) were immunoreactive in either nNOS null or transgenic cultures. The very faint staining seen in nNOS null transgenic cultures is likely to be an artifact of cell clusters. Scale bar, 100 μ m. *e*, Glutamate/depolarization conjunctive stimulation (indicated by horizontal bar at $t = 0$ min) resulted in LTD induction that was apparent in the first test pulses after conjunction ($62.0 \pm 9.3\%$ of baseline at $t = 5$ min, $n = 6$ cells) and persisted for the duration of the recording ($56.5 \pm 8.9\%$ at $t = 45$ min). *f*, Representative current traces corresponding to the times indicated on the graph in *e*. Each trace is a single response and all traces were recorded in the same cell. Calibration: 1 sec, 50 pA.

Figure 1. Continued.

(Roskams et al., 1994). Immunostaining was visualized with biotin-conjugated secondary antibody (goat anti-rabbit) and an avidin-biotin-peroxidase complex (Vector Elite, Vector Laboratories) using diaminobenzidine as the substrate. Wild-type and nNOS null cultures were subjected to identical immunostaining protocols.

Results

Figure 1 illustrates an experiment in which LTD was induced in cultured PN from nNOS null transgenic mice. After acquisition of 10 min of baseline responses, LTD was induced by pairing six successive glutamate pulses with six 3 sec voltage steps to +10 mV, timed so that the depolarization onset preceded the glutamate pulse by 500 msec. LTD induced in these cells was indistinguishable from that in PN cultured from wild-type embryonic mice (compare with Fig. 4 and with examples in Linden and Connor, 1992, or Linden, 1994a). In two cases, recordings were maintained until $t = 120$ min, at which point the glutamate current amplitudes were 51.0 and 62.7% of baseline, suggesting that neither an early nor a later phase of LTD was altered by nNOS knockout. In two additional PN, LTD was induced by three, rather than six glutamate/depolarization pairings (64.9 and 58.8% of baseline current amplitude at $t = 45$ min). nNOS immunoreactivity in these cultures was examined using an affinity purified polyclonal antiserum directed against a glutathione-S-transferase fusion protein containing amino acids 1–181 of nNOS (Roskams et al., 1994). Note that the immunoreactivity seen in wild-type cultures, particularly in small, round clustering presumptive granule cells, is absent in cultures from nNOS null mice (Fig. 1). No nNOS immunoreactive PN were seen in either wild-type or nNOS null cultures. Immunoreactivity for cGMP-dependent protein kinase was assessed using an antiserum directed against this protein from rat (kindly provided by Drs. U. Walter and P. De Camilli), and showed strong signal in both wild-type and nNOS null cultures (data not shown).

Figure 2 illustrates experiments designed to evaluate the effects of cGMP and cGMP analogs on glutamate-evoked inward

currents applied using the same test pulses protocol as in LTD experiments. Both cGMP, which is not readily permeable to cell membranes, and 8-CPT-cGMP, which is, produced a large, transient attenuation of glutamate-evoked currents (24.5 ± 5.8 and $27.3 \pm 5.1\%$ of baseline, respectively, at $t = 5$ min, $n = 5$), which completely recovered to baseline values upon washout (102.0 ± 7.1 and $99.3 \pm 6.3\%$ at $t = 25$ min). This effect did not appear to be dependent upon an interaction between cGMP analogs, and the glutamate test pulses as cGMP applied in the absence of glutamate test pulses (from $t = 0$ –5 min) gave rise to a similar degree of attenuation evident in the first test pulse when resumed after cGMP washout (23.8 and 65.6% of baseline at $t = 6$ and 9 min, respectively). To determine if the effect of cGMP analogs was being exerted internally or at the cell surface, several manipulations were performed. First, inclusion in the patch pipette saline of KT5823, a PKG inhibitor (Kase, 1988), did not block the transient depression produced by cGMP analogs. Second, two manipulations that should raise cGMP levels inside the cell, bath application of protoporphyrin-IX, a membrane permeable activator of soluble guanylate cyclase (Ignarro et al., 1992) or zaprinast, a membrane permeable inhibitor of type V cGMP phosphodiesterase (Burns et al., 1992), both failed to attenuate glutamate-evoked currents. Third, inclusion of cGMP, or its slowly metabolized analog Sp-cGMP-S, in the recording pipette saline did not give rise to a slowly developing attenuation of glutamate-current amplitude as might be expected if cGMP were acting internally.

As one model for the role of NO/cGMP signaling in cerebellar LTD induction depends upon the formation of cADPr and the consequent potentiation of Ca-induced Ca release (Berridge, 1993), we investigated the effect of internally applied cADPr and externally applied NO donors on the Ca signals evoked by a 3 sec depolarizing step to +10 mV (Fig. 3a). This depolarization protocol was chosen because it is the same one employed in our LTD induction protocol. cADPr produced several effects, a small potentiation of the basal Ca level recorded before depolarization, and a larger potentiation of the total Ca increase evoked by the depolarizing pulse caused by both an increase in peak Ca and a decrease in the rate of recovery to baseline. This effect was not mimicked by the NO donors sodium nitroprusside (SNP, Fig. 3b) or *S*-nitroso-*N*-acetyl-penicillamine (100 μ M, $n = 2$, data not shown), nor by the soluble guanylate cyclase activator, protoporphyrin-IX. Furthermore, the effect of cADPr could be completely antagonized by co-inclusion of 8-NH₂-cADPr, an inhibitor of the potentiating effect of cADPr on Ca-induced Ca release (Walseth and Lee, 1993). 8-NH₂-cADPr applied alone had no effect on basal Ca but significantly reduced both the peak and total Ca increase evoked by depolarization (75.1 ± 4.5 and $67.0 \pm 6.3\%$ of baseline, respectively, $n = 4$ /group).

Finally, as one major target of NO-mediated cGMP production is PKG, an enzyme that is highly enriched in PN as determined by immunocytochemistry both *in vivo* (De Camilli et al., 1994) and in our cultures (data not shown), we chose to determine if inhibitors of PKG could alter LTD induction. Internal application of three different PKG inhibitors, KT5823, RKRARKE peptide (Glass, 1983), and Rp-8-CPT-cGMP-S (Butt et al., 1990), all failed to alter LTD induced by glutamate/depolarization conjunction (Fig. 4). In addition, internal application of 8-NH₂-cADPr at the same concentration that completely blocked the effect of exogenous cADPr (2 μ M), failed to alter LTD induction. Internal application of a PKC inhibitor, cheler-

Figure 2. A transient attenuation of glutamate responses by cGMP analogs is produced by an action at the cell surface. *a*, External application of cGMP (1 mM, from $t = 0$ –5 min, indicated by the horizontal bar) or 8-CPT-cGMP (1 mM) gave rise to a transient attenuation of glutamate-evoked inward currents. External application of protoporphyrin IX (PP-IX, 5 μ M) or zaprinast (2 μ M) produced a small potentiation. The attenuation produced by externally applied cGMP or 8-CPT-cGMP was not blocked by inclusion of the PKG inhibitor KT5823 (1 μ M) in the recording pipette saline. Each case is illustrated by a recording from a single representative PN. *b*, Current traces from the protoporphyrin IX and 8-CPT-cGMP treatments corresponding to the times indicated on the graph in *a*. Calibration: 1 sec, 60 pA. *c*, When cGMP (1 mM, $t = 0$ –5 min) was applied without glutamate test pulses, resumption of test pulses at either $t = 6$ min or $t = 9$ min revealed a similar degree of attenuation to that seen when glutamate pulses were applied together with cGMP. *d*, Mean $\pm$ SEM for the cases illustrated by representative single PNs in *a*. In addition, inclusion of cGMP (0.5 mM) or Sp-cGMP-S (0.5 mM) in the recording pipette saline did not give rise to attenuation of glutamate-evoked currents. $n = 5$ /group.

ythrine (Herbert et al., 1990) blocked LTD induction and caused glutamate/depolarization conjunction to result in a small potentiation, in a manner similar to previous experiments with other PKC inhibitors (Linden and Connor, 1991).

Discussion

The finding that LTD induced by glutamate/depolarization conjunction in PNs from nNOS knockout mice was indistinguishable from that in wild-type controls argues strongly that activation of this enzyme is not required for LTD induction in a cell culture system. Furthermore, the observation that in the wild-type mouse both NOS activity, as indicated by the diaphorase stain (Schilling et al., 1994), and nNOS immunoreactivity (Fig. 1) are present in the same cell types in the present culture system as seen in the intact cerebellar cortex *in vivo* suggests that the expression pattern of this enzyme is not significantly altered by our culture protocol. However, these experiments do not address the possibility of eNOS involvement in LTD induction, an issue that has arisen in consideration of a role for NO in the induction of hippocampal homosynaptic LTP (Dinerman et al., 1994).

The experiments illustrated in Figure 2 indicate that the effect of externally applied cGMP analogs on glutamate-gated inward currents is transient and due to an action at the cell surface rather than activation of a cytosolic enzyme. This might be similar to the reported effects of cAMP and its analogs at the GABA_A receptor-associated ion channel of cultured hippocampal neurons, which are also transient, mediated by action at an extracellular site, and operative at millimolar concentrations (Lambert and Harrison, 1990). In addition, the present effect might underlie the transient depression of PN spiking activity recorded extracellularly in response to iontophoresis of cGMP (Hoffer et al., 1971; Siggins et al., 1976).

Is it possible that extracellular actions of cGMP on glutamate receptors might be a physiologically relevant process in the intact cerebellum? An efflux of cGMP from cerebellar slices may be detected in response to glutamate or high K (Tjörnhagmar et al., 1986), and a microdialysis probe inserted in the cerebellum of awake, behaving rats can record an increase in extracellular cGMP stimulated by NO donors or climbing fiber activation (Vallebuona and Raiteri, 1993; Luo et al., 1994). This efflux is potentiated by drugs that block anion transport (probenecid,

Figure 3. cADPr, but not an NO donor, produces an increase in both basal and depolarization-evoked free intracellular Ca. *a*, The time course illustrating basal Ca levels and Ca increases evoked by a 3 sec depolarizing voltage step from -80 to $+10$ mV with control internal saline, internal saline containing cADPr ($0.5 \mu\text{M}$) and internal saline containing both cADPr ($0.5 \mu\text{M}$) and $8\text{-NH}_2\text{-cADPr}$ ($2 \mu\text{M}$). Each case is illustrated by a recording from a single representative PN. *b*, Mean $\pm$ SEM for the cases illustrated by representative single PNs in *a*. Inclusion of $8\text{-NH}_2\text{-cADPr}$ ($2 \mu\text{M}$) in the recording pipette had no effect on basal Ca, but produced a small depression of depolarization-evoked Ca. In addition, external application of Na nitroprusside (SNP, 0.3 or 3 mM) or protoporphyrin-IX (PP-IX, $5 \mu\text{M}$) starting at $t = 5$ min had no effect on either basal or depolarization evoked Ca. The basal measure was taken as the mean of the seven values recorded from $t = -3$ to 0 sec. Peak measures were taken at $t = 1$ sec, which was always the time point with the largest response. The sum measure represents the integrated Ca increase over the period $t = 0$ – 12 sec. $n = 4$ /group.

bromosulphophthalein) suggesting that the removal of cGMP from the extracellular space is dependent upon such a mechanism (Luo et al., 1994). However, it is not clear if the concentration of cGMP achieved extracellularly in the intact cerebellum, estimated to be approximately 10 – 200 nM is ever large enough to produce the effects seen in the present study using 1 mM cGMP. Pilot studies using extracellular application of $1 \mu\text{M}$ cGMP did not produce any attenuation of glutamate-evoked currents (data not shown).

Ca-induced Ca release mediated by ryanodine receptors has been demonstrated in PNs as a supralinear internal Ca response evoked by depolarizing voltage-clamp commands of increasing

Figure 4. Inhibitors of PKG or the cADPr/ryanodine receptor interaction do not alter cerebellar LTD. *a*, Neither the PKG inhibitors KT5823 ($1 \mu\text{M}$), Rp-8CPT-cGMP-S (1 mM), RKRARKE peptide (0.5 mM), nor the cADPr antagonist $8\text{-NH}_2\text{-cADPr}$ ($2 \mu\text{M}$) blocked LTD induced by glutamate/depolarization conjunction (indicated by horizontal bar at $t = 0$ min) when included in the pipette saline. Inclusion of the protein kinase C inhibitor chelerythrine ($10 \mu\text{M}$) in the pipette saline completely blocked LTD induction. $n = 6$ /group. *b*, Representative current traces. Calibration: 1 sec, 40 pA.

lengths in TTX saline. The supralinear portion of the response, which was presumed to represent Ca-induced Ca release (riding on top of a signal attributable to Ca influx), was potentiated by ryanodine and inhibited by a ryanodine receptor antagonist, ruthenium red (Llano et al., 1994). Furthermore, in bullfrog sympathetic ganglion cells it has been shown that internal application of cADPr augments depolarization-induced increases in intracellular Ca, and that this augmentation may be blocked by ryanodine (Hua et al., 1994). The present results confirm and extend these observations by demonstrating that the Ca response to a prolonged depolarizing pulse in PNs may be potentiated by cADPr, presumably acting at the ryanodine receptor. Furthermore, the observation that $8\text{-NH}_2\text{-cADPr}$ blocks a portion of the depolarization-induced response indicates that cADPr may function as an endogenous modulator of Ca signaling in PNs.

Internal application of three different PKG inhibitors failed to alter LTD induction. This result is in contrast with the findings of Hartell (1994), who reported that intracellular application of KT5823 (90 nM) entirely blocked LTD induced by parallel fiber/climbing fiber conjunctive stimulation. Furthermore, our finding that the same dose of $8\text{-NH}_2\text{-cADPr}$ that completely blocked the cADPr-mediated increase in Ca, failed to alter LTD induction argues that this process, while operative in cultured PNs, is not necessary for LTD induction.

Taken together with the previous observation that LTD of glu-

tamate currents was unaffected by NOS inhibitors, hemoglobin, or an NO donor (Linden and Connor, 1992), the present findings do not support a role for any portion of a NO/cGMP/PKG cascade in the induction of cerebellar LTD in culture, as envisaged by the first two models of NO/cGMP action discussed in the Introduction. There are several potential explanations for this contradiction, none of which is completely satisfactory. One is that the difference lies in the type of stimulus used to monitor LTD. Investigations from other laboratories have used parallel fiber stimulation to monitor LTD in the cerebellar slice, while our study used glutamate pulses applied to PN in culture. Therefore, if the target of NO/cGMP action were a site other than the PN dendrite (such as the parallel fiber terminal), then the effect of NO/cGMP reagents might be expected to be observed with PF stimulation used as the test stimulus but not with glutamate pulses used in the present study. However, two observations suggest that this explanation might not be correct. First, internal application of cGMP to PN in cerebellar slices produces an LTD-like effect when combined with parallel fiber stimulation (Daniel et al., 1993; Hartell, 1994). Similarly, internal application of PKG inhibitors but not a NOS inhibitor blocked induction of LTD, suggesting a model (similar to the first two in the Introduction) in which NO formed outside of the PN activates guanylate cyclase within the PN to contribute to LTD induction in the PN in slice. Second, one report using the slice preparation showed no effect of a bath-applied NO donor on PF-evoked EPSPs recorded in Purkinje neurons (Glaum et al., 1992).

Another explanation that was not directly addressed by the present study is that NO produced in the PN by climbing fiber stimulation blocks a tonic signal from neighboring glial cells that suppresses LTD induction. This model was suggested by the observation that treatment of cultured cerebellar slices with a gliotoxic metabolic inhibitor converted LTD from an NO-dependent (hemoglobin and L-monomethylarginine sensitive) form to an NO-independent form (Shibuki, 1993). As an explanation for the present results, this model requires that this glial suppression not be operative in cerebellar cultures. While the cultures are well endowed with glia as assessed by GFAP immunoreactivity (data not shown), it is, nonetheless, possible that the association between the relevant glial cells and the PN is less intimate in culture than in an acute cerebellar slice. Unfortunately, the glial suppression hypothesis is not easily testable using either slice or culture preparations as gliotoxins (such as fluorocitrate) are quite nonspecific.

Finally, it has been suggested that PN in culture fail to develop some cellular machinery that confers upon them the ability to respond to NO released from parallel fibers (Crepel et al., 1994). However, since PN in culture from both wild-type and nNOS null mice readily demonstrate LTD, it would appear that such a developmental failure, if it, indeed, occurs, is not sufficient to prevent this form of plasticity. However, this does not mean that such a requirement could not be present in the cerebellar slice.

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