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Long-Term Depression in Cerebellar Purkinje Neurons Results from Coincidence of Nitric Oxide and Depolarization-Induced Ca^{2+} Transients

Varda Lev-Ram,* Lewis R. Makings,*† Paul F. Keitz,‡ Joseph P. Y. Kao,‡ and Roger Y. Tsien*§
*Department of Pharmacology
§Howard Hughes Medical Institute
University of California, San Diego
La Jolla, California 92093-0647
‡Medical Biotechnology Center
Department of Physiology,
University of Maryland School of Medicine
Baltimore, Maryland 21201

Summary

The role of nitric oxide (NO) in the induction of long-term depression (LTD) in the cerebellum was explored using a new, organic, membrane-impermeant form of caged NO. NO photolytically released inside Purkinje neurons mimicked parallel fiber (PF) activity in synergizing with brief postsynaptic depolarization to induce LTD. Such LTD required a delay of <50 ms between the end of photolysis and the onset of depolarization, was prevented by intracellular Ca^{2+} chelation, and was mutually occlusive with LTD conventionally produced by PF activation plus depolarization. Bath application of NO synthase inhibitor or of myoglobin, a NO trap, prevented LTD induction via PF stimulation, but not that from intracellular uncaged NO, whereas intracellular myoglobin blocked both protocols. NO is therefore an anterograde transmitter in LTD induction. A biochemical requirement for simultaneous NO and elevation of intracellular free Ca^{2+} would explain why PF activity must coincide with postsynaptic action potentials.

Introduction

Long-term changes in synaptic efficacy are thought to be the neuronal bases for learning and memory. Long-term depression (LTD) of synaptic transmission between parallel fibers (PFs) and Purkinje cells (PCs) in the cerebellum is one of the most important cellular models for acute synaptic plasticity in the vertebrate central nervous system. Its fundamental importance for associative learning and classical conditioning has been advocated (Ito, 1986; Thompson, 1990) and questioned (Llinás and Welsh, 1993; Schreurs and Alkon, 1993). LTD can be induced in intact animals or in vitro cerebellar slices by costimulation of climbing fibers and PFs (Ito et al., 1982; Sakurai, 1987; Crépel and Jaillard, 1991). Climbing fiber activation normally triggers an action potential in the PC and can be replaced by depolarization of the PC, which induces Ca^{2+} influx (Konnerth et al., 1992). Thus, postsynaptic depolarization coincident with presynaptic PF stimulation is sufficient for LTD induction (Crépel and Krupa, 1988; Konnerth et al., 1992). LTD is probably mediated by down-regulation of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropimate (AMPA) receptors at PF–PC synapses (Hémart et al., 1994; Linden and Connor, 1995). LTD induction has been argued to require activation of both metabotropic and ionotropic Purkinje glutamate receptors (Ito and Karachot, 1990). However, in cerebellar slices, but not in cultured Purkinje neurons, activation of the metabotropic glutamate receptor in conjunction with depolarization is sufficient to induce LTD (Daniel et al., 1992; Linden et al., 1993).

Nitric oxide (NO) has been suggested to play an important role in several models of neuronal plasticity (for review, see Schuman and Madison, 1994). In the cerebellum, NO synthase (NOS) is abundant in granule, basket, and Bergman glia cells (Bredt et al., 1990), thus pointing to a possible role for NO in cerebellar communication. Indeed, NO participation in LTD induction has been both advocated (Ito and Karachot, 1990; Shibuki and Okada 1991; Crépel and Jaillard, 1990; Daniel et al., 1993) and downplayed (Glaum et al., 1992; Linden and Connor, 1992). Even if NO is involved, its source(s) and target(s) are still controversial, some reviewers assigning NO to be produced by climbing fiber stimulation (Shibuki and Okada, 1991; Hartell, 1994; Linden and Connor, 1995) and others considering it to originate from the PF pathway (Schuman and Madison, 1994), whose cell bodies are the granule cells.

Previous investigations of the role of NO in cerebellar LTD have relied on pharmacological inhibitors of NOS or on molecules that release NO in a spatially and temporally uncontrolled manner. Inhibitors are useful but potentially nonspecific; thus, careful controls are needed. The best control for a NOS inhibitor is to show that its effect is circumvented by appropriate delivery of NO. Because any actions of NO in synaptic plasticity are likely to be local, specific for particular cell types, and transient, we need comparable experimental control of NO delivery. Global steady-state NO donors may be as unrealistic as global steady-state incubation with neurotransmitters such as glutamate. Therefore, we have used newly synthesized, totally organic molecules that release NO upon photolysis. The release of NO proceeds with adequate quantum efficiency upon illumination with near UV light and is complete within 5 ms of the end of a flash (Makings and Tsien, 1994). Related members of the family offer high, low, or temporary permeability through membranes; we chose to use CNO-4, which is highly polar and membrane impermeant because it has two negative charges. CNO-4 as its dipotassium salt was introduced into individual Purkinje neurons in acutely prepared rat cerebellar slices by patch clamping in the whole-cell configuration. We found that photolytic generation of NO could completely replace PF activation in the induction of LTD. Additional experiments with NO traps and NOS inhibitors suggest that PF stimula-

†Present address: Cypros Pharmaceutical Corporation, 2732 Loker Avenue, West Carlsbad, California 92008.

tion generates NO outside the Purkinje neuron, that this NO is required for LTD, and that the NO target(s) resides inside Purkinje neurons, which implies that NO is an essential anterograde messenger for LTD induction.

Results

Simultaneous Postsynaptic Depolarization and Uncaging of NO Induce LTD

Photolytic release of NO from membrane-impermeant CNO-4 inside the PC, in conjunction with depolarization-induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) transients, reliably induced LTD of the PF–PC synapse (Figure 1). The same whole-cell patch pipette on the PC delivered the CNO-4 and depolarization as well as recorded the synaptic currents. A 5–10 min baseline of stable excitatory postsynaptic current (EPSC) amplitude was followed by a protocol of brief depolarization (50 ms) in conjunction with near UV light exposure (900 ms) for 10 s at 1 Hz. As few as 8 repetitions were often sufficient, though in many other experiments 30 repetitions at 1 Hz were delivered to ensure consistent LTD induction. After this training period, LTD was observed in 94% of cells (n = 48). The LTD consistently occluded any further reductions due either to additional training periods or to conventional PF–PC costimulation.

Both photorelease of NO and PC depolarizations were required to induce LTD. When CNO-4 was omitted and the cell was exposed to 30 light flashes of 900 ms duration at 1 Hz by themselves (data not shown), or in conjunction with depolarizing pulses (Figure 2), the EPSCs were not influenced, though the ability of the synapses to undergo LTD was confirmed by subsequent PF–PC costimulation. Conversely, photolysis of CNO-4 without depolarization did not induce LTD even when PF stimulation was supplied concurrently (Figure 3). Nevertheless, the synapse subsequently displayed LTD once uncaged NO was properly paired with PC depolarization. Induction of LTD by NO plus depolarization required a rise in PC $[Ca^{2+}]_i$, because it was blocked by the Ca^{2+} chelator BAPTA at 5 mM in the patch pipette (Figure 4). The specific need for synergism with the depolarization-induced $[Ca^{2+}]_i$ rise also indicates that the reduction in EPSC amplitude was not due to nonspecific photodynamic damage or NO-induced toxicity to the participating cellular elements.

Coincidence Timing Requirements for NO and Depolarization

Caged compounds are uniquely advantageous for delivering messenger substances with temporal precision. CNO-4 belongs to a family of molecules whose release of NO tracks the time course of illumination with less than 5 ms delay (Makings and Tsien, 1994). Such fast kinetics permit exploration of the coincidence requirement for NO and depolarization to induce LTD. Figure 5 shows that LTD was induced if the depolarization began 0 but not 50 or 175 ms after the end of photolytic generation of NO.

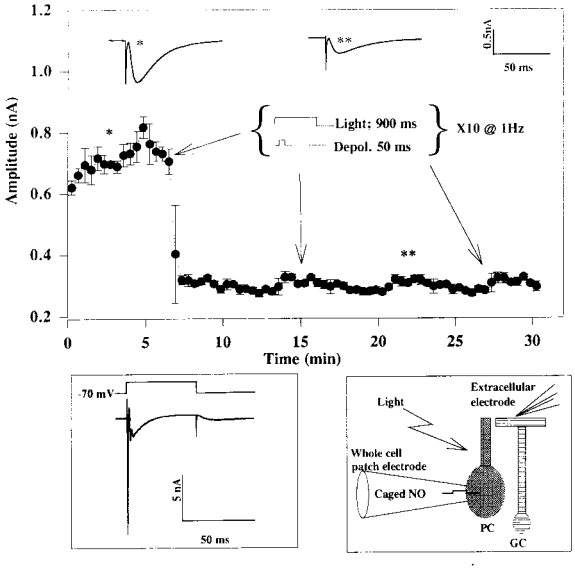

Figure 1. Uncaged NO and Depolarization Together Induce LTD

Caged NO (200 μM CNO-4, di-K^+ salt) was introduced into a cerebellar Purkinje neuron using the whole-cell patch-clamp technique. Each ordinate is the average of the peak amplitudes of five consecutive EPSCs induced by PF stimulation at 0.2 Hz. Error bars represent the SD of the five amplitudes. After 6 min of baseline recording, dim UV (900 ms; see Experimental Procedures) and direct depolarization of the cell (50 ms centered within the illumination period) were applied 10 times at 1 Hz. This combination resulted in an abrupt, long-lasting decrease of the EPSC amplitudes by >50%. Two additional UV–depolarization episodes (marked by arrows) had no additional effect. The insets marked with asterisks are traces with expanded time resolution showing averages of ten consecutive EPSCs, sampled before and after induction of LTD as marked on the main record. The initial sharp, downward deflection in each inset is the stimulus artifact. LTD was induced in 45 out of 48 cells treated as in this figure. The bottom left panel shows a sample of the current recorded during the 50 ms depolarizing pulse. This trace was taken from a different cell because it required lower amplifier gain.

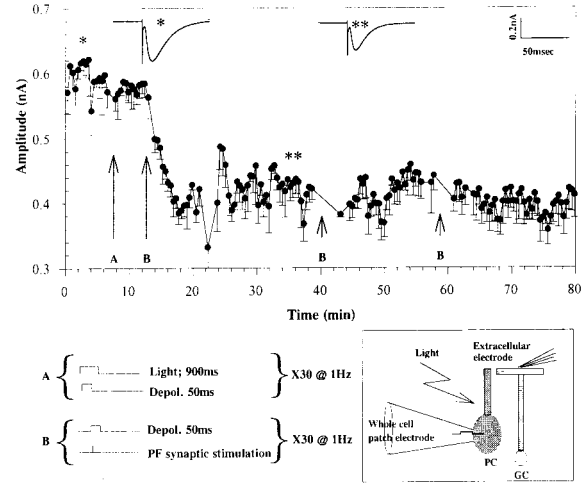

Figure 2. Light and Depolarization Do Not Induce LTD in the Absence of CNO-4

A typical control experiment similar to that in Figure 1, except that CNO-4 was omitted from the patch pipette. The combination of simultaneous dim UV and depolarization did not induce LTD (stimulus A). As a positive control, depolarization and PF stimulation were simultaneously applied 30 times at 1 Hz where indicated (stimulus B), resulting in LTD. This experiment worked in 4 out of 4 cells tested.

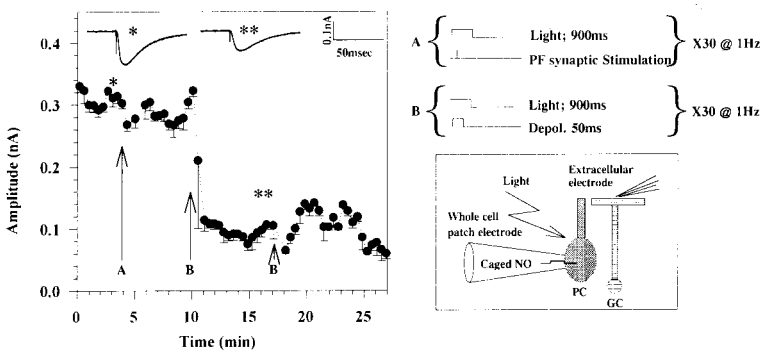

Figure 3. Uncaged NO Does Not Synergize with PF Activity to Induce LTD

When NO was photoreleased by dim UV simultaneously with PF stimulation (stimulus A), there was no significant change in the EPSCs. As a positive control 5 min later, simultaneous uncaging of NO and depolarization (stimulus B) produced LTD, which could not be augmented by further repetition (stimulus B). This experiment was typical of 4 out of 5 cells tested.

The clear implication is that an effective concentration of NO lasts for significantly less than 50 ms after NO generation terminates. When the order of stimuli was reversed, so that depolarization preceded NO generation, the coincidence requirements were slightly less stringent. LTD was induced if photolysis began within 100–150 but not 200 ms after the end of the depolarization (Figure 6). This difference between the reciprocal experimental protocols implies that in PCs NO has a shorter duration of biochemical efficacy than the depolarization-induced elevation of $[Ca^{2+}]_i$ (Lev-Ram et al., 1992). Previous workers have obtained varying requirements for optimal timing of PF activity and depolarization to produce LTD, but there is agreement that the two physiological inputs need to coincide within 50–250 ms (reviewed by Linden and Connor, 1995).

Sources and Targets of NO for LTD Induction

The experiments described above establish the efficacy of exogenous NO. To examine the importance, sources, and targets of endogenous NO in LTD induction, a NOS inhibitor, N^G-nitro-L-arginine, and a NO scavenger, myoglobin, were applied extra- and intracellularly. N^G-nitro-L-arginine is an arginine analog taken up into cells and should globally inhibit NOS when applied extracellularly at 50–100 μM for 1–4 hr (East and Garthwaite, 1990; Cum-

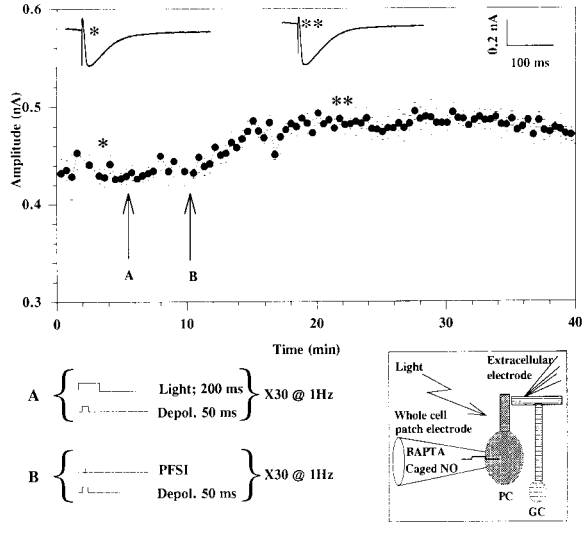

Figure 4. Intracellularly Applied BAPTA Prevents LTD Induction by either PF Stimulation or Uncaged NO

CNO-4 (200 μM) and BAPTA (5 mM) were included in the patch pipette. Neither uncaging of NO (stimulus A; 200 ms bright UV; see Experimental Procedures) nor PF stimulation (stimulus B), each synchronized with depolarization and repeated 30 times at 1 Hz, could induce LTD. Similar results were obtained in 6 out of 6 cells.

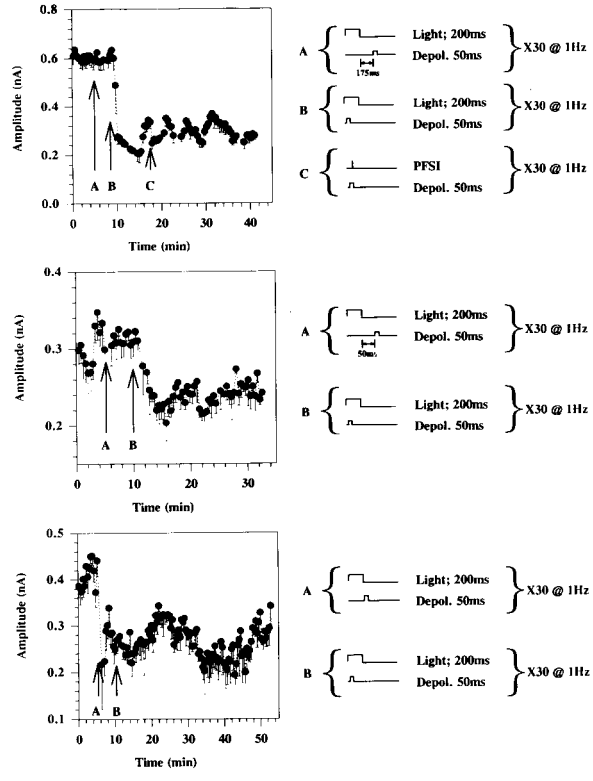

Figure 5. LTD Induction Requires a Gap of Less Than 50 ms between Cessation of NO Release and Onset of Depolarization

A gap of 175 ms (top panel, stimulus A) or 50 ms (middle panel, stimulus A) between the end of a 200 ms period of bright UV and the onset of depolarization, all repeated 30 times at 1 Hz, prevented LTD induction. As positive controls, subsequent simultaneous depolarization and illumination (top and middle panels, stimulus B) induced LTD, which occluded depolarization paired with PF activity (top panel, stimulus C). Depolarizing the Purkinje neuron with no delay after the end of the light period (bottom panel, stimulus A) did induce LTD, which could not be enhanced by additional episodes with better simultaneity (stimulus B). In all 9 experiments with a 100 ms gap, stimulus A failed to give LTD, but stimulus B failed in 3 out of those 9. In 3 experiments with a 50 ms gap and 7 experiments with no gap, all worked as shown.

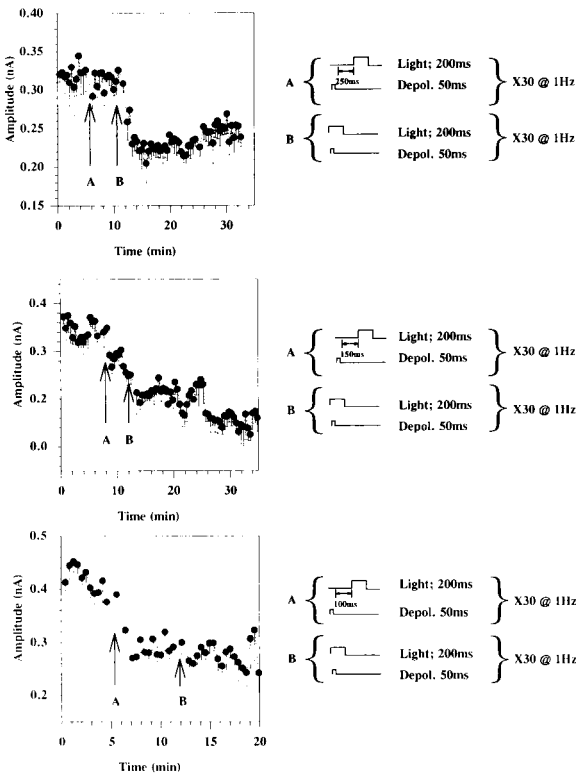

Figure 6. LTD Induction Requires a Gap of Less Than 100–150 Between Cessation of Depolarization and Onset of NO Release

A gap of 250 ms (top panel, stimulus A) or 150 ms (middle panel, stimulus A) between the end of a 50 ms depolarization and the onset of bright UV, all repeated 30 times at 1 Hz, prevented full LTD induction. As positive controls, subsequent simultaneous depolarization and illumination (top and middle panels, stimulus B) induced LTD. In all 5 experiments with a 250 ms gap, stimulus A failed to elicit LTD, but in 1 of those, stimulus B also failed. In 4 out of 5 experiments with a 150 ms gap, stimulus A produced only partial LTD (as in the middle panel) or no effect, while stimulus B was effective; in the fifth cell, stimulus A produced full LTD. With a 100 ms gap, 2 out of 4 cells showed full LTD after stimulus A (as in the bottom panel), while the remaining 2 responded only to stimulus B. In 3 out of 3 experiments with a 50 ms gap (data not shown), stimulus A sufficed to produce full LTD.

mings et al., 1994). PF–PC costimulation was then unable to induce LTD (Figure 7), confirming the findings of Daniel et al. (1993). However, in the very same cell, CNO-4 photolysis, which generates NO by means independent of NOS, still combined with depolarization to induce LTD. These results argue that N^G-nitro-L-arginine at this dose is indeed acting as a specific inhibitor of NOS, and that NOS activity is essential for PF–PC costimulation to induce LTD. Is the relevant NOS within the PC? N^G-nitro-L-arginine blocks NOS with an IC_{50} of 1 μM or less (Dwyer et al., 1991; East and Garthwaite, 1990; Furfine et al., 1993), so inclusion of 5 μM N^G-nitro-L-arginine in the patch pipette should block NOS nearly completely in the PC being patch clamped. N^G-nitro-L-arginine as a zwitterion should not leak out of the cell passively and induce inhibition on neighboring cells. Indeed, PF–PC costimulation induced LTD normally (Figure 8), a result suggesting that such LTD does not need much if any NOS activity within the PC.

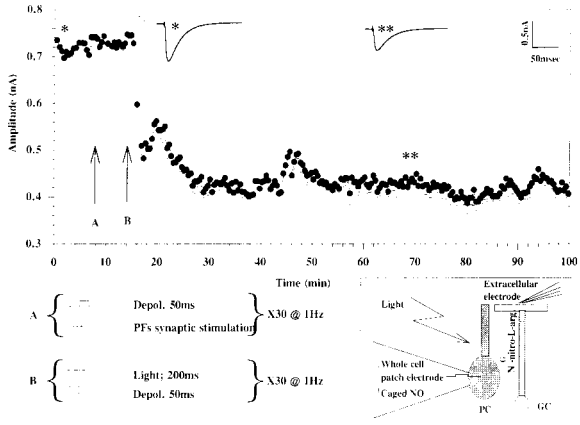

Figure 7. Bath-Applied NOS Inhibitor Blocks LTD from PFs but Not from Uncaged NO

Incubation of the slices in the NOS inhibitor N^G-nitro-L-arginine (100 μM) for 1–4 hr prior to the experiment prevented induction of LTD by PF stimulation paired with depolarization (30 repetitions at 1 Hz; stimulus A). As a positive control, LTD was induced by simultaneous uncaging of NO and depolarization (30 repetitions at 1 Hz; stimulus B). In 7 cells tested with the NOS inhibitor, none gave LTD from PF stimulation, while 5 showed LTD from uncaged NO.

N^G-nitro-L-arginine (50 μM) in the patch pipette prevented LTD induction by both protocols (data not shown), pointing to the possibility of nonspecific actions of the inhibitor at high concentrations.

NO-trapping molecules can give valuable complementary clues as to the site of action of NO. We chose oxymyoglobin (MbO_2) as a NO trap because it has high affinity for NO, is biocompatible and membrane impermeant, and has one-fourth the molecular weight of the more commonly used hemoglobin. After establishing the whole-cell patch-clamp configuration on a PC, 10 μM MbO_2 was added to the normal Ringer's solution (Figure 9). This dose of extracellular MbO_2 had no effect on the baseline amplitude of the PF EPSCs, but PF–PC costimulation failed to induce LTD. However, intracellular uncaging of NO still combined with depolarization to produce LTD in the same PC. These results argue that induction of LTD by PF–PC costimulation requires that NO cross an intercellular gap, where it can be intercepted by extracellular MbO_2. The necessary target for such NO is inside the PC, because intracellularly uncaged NO is still effective. We have previously shown in blood platelets that, when NO is generated and sensed in the very same cell, extracellular NO traps cannot block such local action (Makings and Tsien, 1994).

If the target of NO is inside the PC, one would predict that intracellular MbO_2 should prevent both PF stimulation and NO uncaging from inducing LTD. Because PCs contain 3–30 μM guanylate cyclase (M. Nakane, personal communication) and MbO_2 has to diffuse from the patch pipette into the cell, 100 μM MbO_2 was included in the patch pipette. As predicted, neither costimulation protocol induced LTD (Figure 10). This result not only confirms that the NO target(s) is within the PC but also provides additional proof that the effects of CNO-4 photolysis are mediated by NO rather than by any side products.

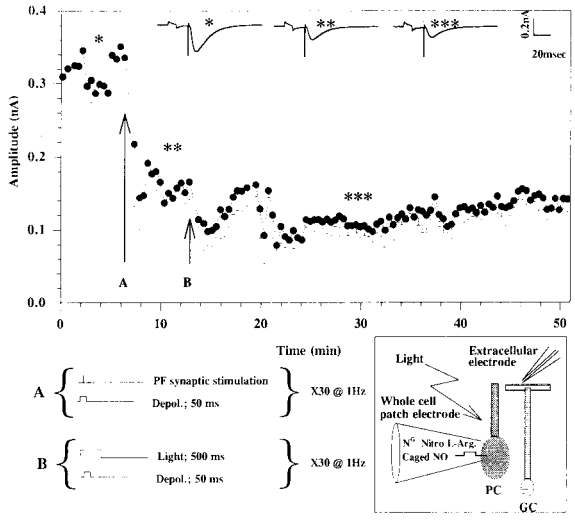

Figure 8. Intracellularly Applied NOS Inhibitor Does Not Block LTD Induction by PFs

CNO-4 (200 μM) and N^G-nitro-L-arginine (5 μM) were introduced into the PC via the patch pipette. Simultaneous PF stimulation and depolarization (30 repetitions at 1 Hz; stimulus A) produced LTD, which could not be significantly enhanced by subsequent uncaging of NO and depolarization (30 repetitions at 1 Hz; stimulus B). In this experiment, a 10 ms (10 mV) test depolarization preceded the PF synaptic stimulation, as visible in the insets at expanded time resolution. The serial resistance and capacitance compensation were turned off to demonstrate that changes in the amplitude of EPSCs were not due to changes in series resistance during the various experimental manipulations. In 7 cells tested, 5 showed LTD due to PF stimulation, while the remaining 2 did not respond to either protocol.

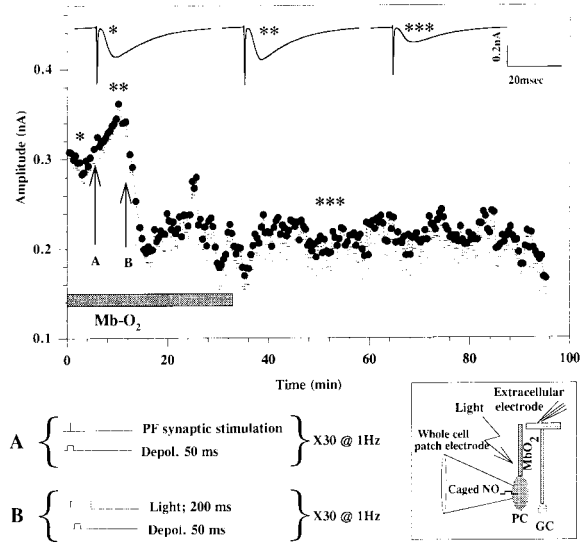

Figure 9. Extracellular Oxmyoglobin Inhibits LTD Induction by PF Stimulation but Not by Uncaged NO

After establishment of the whole-cell patch configuration, MbO_2 (10 μM) was included in the perfusing Ringer's solution for the duration indicated by the shaded bar. PF stimulation together with depolarization (30 repetitions at 1 Hz; stimulus A) failed to elicit LTD, whereas simultaneous uncaging of NO and depolarization (30 repetitions at 1 Hz; stimulus B) was still able to induce LTD. Similar results were obtained in 4 out of 4 cells.

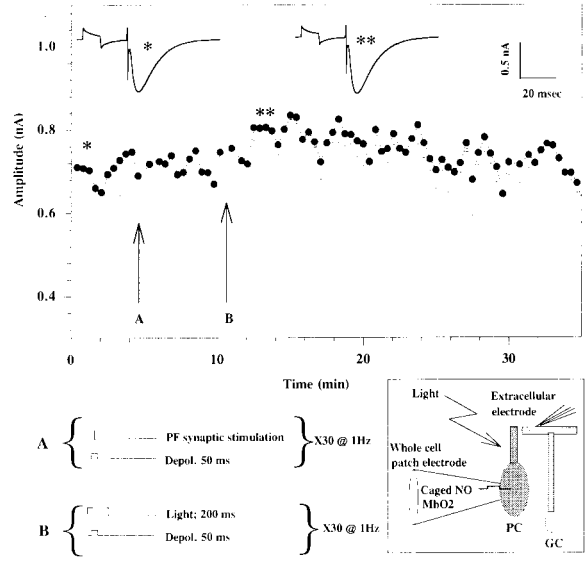

Figure 10. Intracellular Oxymyoglobin Inhibits LTD Induction by either PF Stimulation or Uncaged NO

CNO-4 (200 μM) and MbO_2 (100 μM) were included in the patch pipette. Neither PF stimulation (stimulus A) nor uncaging of NO (stimulus B), each synchronized with depolarization and repeated 30 times at 1 Hz, could induce LTD. In this experiment, as in Figure 8, a 10 ms (10 mV) test pulse was included before each PF synaptic stimulation to demonstrate constancy of series resistance. Similar results were obtained in 4 out of 5 cells, the fifth being too unstable to judge.

Uncaged CO Can Also Synergize with Depolarization to Induce LTD

Carbon monoxide (CO) has been proposed as another gaseous intercellular messenger that can activate guanylate cyclase, and that might participate in long-term potentiation in the hippocampus (Stevens and Wang, 1993; Zhuo et al., 1993). These proposals, together with the recent invention of a caged CO (Figure 11) (Kao and Keitz, 1995, FASEB J., abstract), prompted us to investigate whether CO can replace NO in inducing LTD. The present caged CO, "NV-CO," uses the same photosensitive nitrobenzyl moiety as CNO-4 and also has a negative charge to confer hydrophilicity and hinder transmembrane diffusion. It was included in the patch pipette at the same concentration (200 μM) as is usually employed for CNO-4. Like caged NO, it produced LTD only when photolyzed in conjunction with depolarization (Figure 11). However, much more UV light was required. It is not yet clear whether this reflects a poorer quantum efficiency for photolysis of NV-CO compared with CNO-4 or a lesser biological sensitivity to CO compared with NO.

Discussion

When cerebellar LTD was first described, it was induced by simultaneous stimulation of the PF and the climbing fiber input to the PC. The first reduction in complexity was the demonstration that the climbing fiber input could be replaced by direct depolarization, which probably serves merely to elevate $[Ca^{2+}]_i$ in the PC (Konnerth et al., 1992).

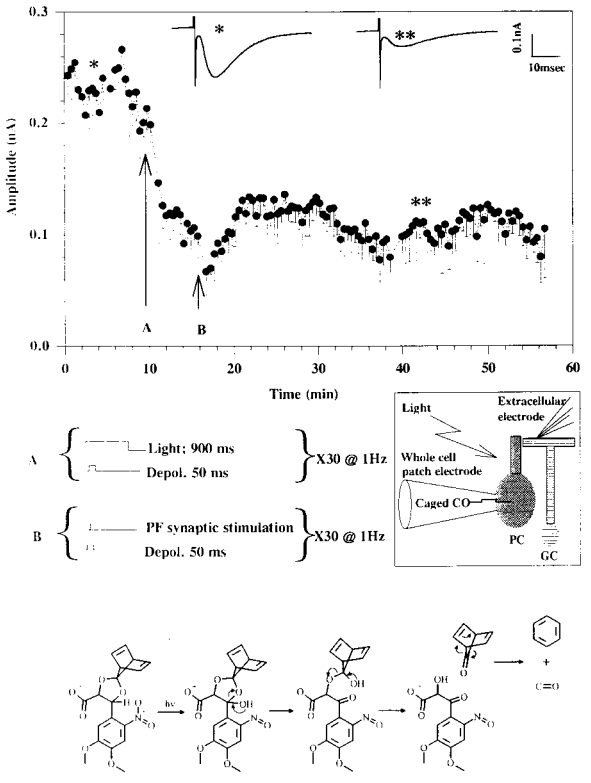

Figure 11. Uncaged CO plus Depolarization Can Induce LTD

(Top) Caged CO (NV-CO, 200 μM) was introduced into the PC by inclusion in the patch pipette. After 10 min of baseline recording, LTD was induced by simultaneous depolarization and illumination with bright UV for 900 ms, repeated 30 times at 1 Hz (stimulus A). Further depolarization and PF stimulation (stimulus B) failed to produce additional LTD. Stimulus A produced LTD in 4 out of 5 cells tested; the fifth failed to respond to stimulus B either. In 2 out of 2 cells, uncaging of CO without depolarization failed to induce LTD (data not shown). (Bottom) The structure of NV-CO is shown at the far left. The first steps after photolysis are the hydrolysis of the ketal that masks the carbonyl group of norbornadienone. Once the latter is revealed, extrusion of CO (last step, upper right) is driven by the concomitant formation of the stable aromatic ring.

Our present results show that PF activity can be analogously replaced by a biochemically simpler trigger, namely NO, when the latter is generated with appropriate time resolution and cell specificity using a newly invented caged NO. Uncaged NO has the same requirement for temporal coincidence with depolarization and gives the same LTD amplitude and the same or higher success rate as traditional PF stimulation. Whereas LTD induction through PF stimulation is prevented by global NOS blockade or extracellular NO trapping, uncaged NO bypasses these inhibitions. Only intracellular NO trapping blocks the effect of uncaged NO as well as PF stimulation. The simplest explanation for these findings is that the role of PF activity in LTD induction is merely to generate NO as an anterograde transmitter sensed inside the PC. This proposal fits perfectly with the known localization of relevant enzymes. NOS is abundant in granule and basket cells but not in PCs (Bredt et al., 1990), whereas PCs contain extremely high levels of guanylate cyclase (Ariano et al., 1982), a major target for NO. Moreover, LTD expression seems to involve kinase-mediated down-modulation of the AMPA-type glutamate receptors on the PC (Hémart et al., 1994; Linden and Connor, 1995), so the final target is in the appropriate compartment.

The literature on cerebellar LTD contains considerable controversy as to whether NO is involved, and if so, whether it needs to coincide with climbing fiber/depolarization-induced $[Ca^{2+}]_i$ transients, PF activity, or neither. One of the earliest studies attempting to deliver NO was that of Shibuki and Okada (1991), who reported that PF stimulation in conjunction with bath-applied sodium nitroprusside (3 mM) or 8-bromo-cGMP produced LTD, which was monitored indirectly by changes in extracellular K^+ concentrations. Nitroprusside by itself had no significant effect. They concluded that NO and cGMP substitute for climbing fiber activation and synergize with PFs, whereas our results indicate that NO can replace PFs, not depolarization or climbing fiber stimulation. Crépel and Jaillard (1990) and Daniel et al. (1993) likewise found that extracellular nitroprusside (8 mM) or intracellular 3-morpholinosydnonimine (SIN-1; 3 mM) caused LTD during ongoing PF stimulation; dependence on the latter was not explicitly tested. These observations may perhaps be explained by more recent findings (Miyakawa et al., 1992; Eilers et al., 1995) that PF stimulation does elevate $[Ca^{2+}]_i$ significantly through local depolarization-induced activation of voltage-gated Ca^{2+} channels, especially when the PC is not voltage clamped, as in the experiments of both Shibuki and Okada (1991) and Daniel et al. (1993). Neither study tested whether depolarization separate from PF activity could synergize with NO delivery. Yet another possibility is that high doses of nitroprusside or SIN-1, perhaps acting through their toxic byproducts, cyanide and peroxynitrite, might elevate $[Ca^{2+}]_i$ to synergize with NO or simply poison the synapse and masquerade as LTD. Because CNO-4 photolysis releases NO without such toxic byproducts and gives LTD that is abrupt, of robust but saturable amplitude, dependent on temporally precise coincidence with $[Ca^{2+}]_i$, and blocked by intracellular myoglobin, its effects are much less likely to represent nonspecific toxicity. Also, use of voltage clamping in the whole-cell patch-clamp configuration optimizes our ability to distinguish biochemical from electrical effects of PF activity. Glaum et al. (1992) pointed out that the specificity of millimolar concentrations of nitroprusside is questionable, but found that 1–3 mM had no effect on PCs, though concurrent depolarization was not applied.

Extracellularly applied NOS inhibitors and NO scavengers (hemoglobin or methylene blue) have given more consistent results, blocking LTD induction by PF–climbing fiber costimulation (Shibuki and Okada, 1991) or PF–depolarization costimulation (Crépel and Jaillard, 1990). Intracellular delivery of a NOS inhibitor through the patch pipette failed to block LTD (Daniel et al., 1993). Our results confirm these findings, though the effectiveness of an intracellular NO trap appears not to have been demonstrated before. Also, our new ability to test whether intracellularly uncaged NO can bypass the inhibitors and scavengers is very helpful in showing their site of action and distinguishing specific from nonspecific effects.

In cultured Purkinje neurons, LTD of glutamate currents induced by synchronized depolarization and application of glutamate was not affected by up to 10 μM N^G-nitro-L-arginine, 10 μM hemoglobin, or 3 mM sodium nitroprusside (Linden and Connor, 1992). NOS inhibitors or NO scavengers might not be expected to have any effect in the absence of presynaptic elements. The nonoptimal NO donor nitroprusside was administered in conjunction with iontophoretic glutamate pulses, mimicking PF activity, while maintaining the neurons under hyperpolarizing voltage clamp. By analogy to our Figure 3, this protocol should not have generated LTD (and did not). Furthermore, neurons cultured from fetal cerebella may differ significantly from PCs in acute slices from more mature animals. Other biochemical characteristics such as the presence of N-methyl-D-aspartate receptors and NADPH-diaphorase activity are known to differ between these preparations.

The known biochemical targets of NO signal transduction (reviewed by Schuman and Madison, 1994; Bredt and Snyder, 1994) include soluble guanylate cyclase, ADP-ribosyltransferase, and certain critical thiols on proteins such as the N-methyl-D-aspartate receptor. Our finding that uncaged CO can mimic NO (see Figure 11) hints that guanylate cyclase is the main or at least a sufficient target. Whether endogenous CO might also play a role is controversial in hippocampus (Stevens and Wang, 1993; Zhuo et al., 1993; Meffert et al., 1994) and remains to be explored in cerebellum. Unfortunately, hemoglobin and myoglobin will trap both NO and CO, and currently available inhibitors of endogenous CO generation by heme oxygenase may also inhibit NOS (Meffert et al., 1994). Caged NO and CO are at least chemically unambiguous. There is much evidence for, and some against, the role of cGMP in cerebellar LTD (reviewed by Linden and Connor, 1995). Additional experiments, which may require new membrane-impermeant versions of caged cGMP, will be needed to clarify whether cGMP plays a crucial role, and if so, whether it lies upstream or downstream of the detection of coincidence with $[Ca^{2+}]_i$ transients.

Other molecules that cage NO have been used to test the hypothesis that NO is a retrograde messenger involved in long-term potentiation in the hippocampus. Uncaged NO caused depressions rather than long-term enhancements in synaptic strength (Boulton et al., 1994; Murphy et al., 1994). These results argue against any simple role for NO in causing long-term potentiation in the CA1 region, but were obtained with iron and ruthenium nitrosyl complexes in which NO release leaves behind coordination sites on these heavy metals. Also, the NO donors were bath applied, so that all cells in the slice should have been indiscriminately exposed. The results obtained in the present study suggest that it might be worth reexamining the role of NO in the hippocampus using CNO-4, which contains no metals and can be loaded into specific neurons.

The narrow time window within which a fast-rising $[Ca^{2+}]_i$ transient can synergize with a previous NO pulse argues strongly that the effective lifetime of NO in PCs is well under 50 ms. Even though 50 ms is only an upper limit, it is the shortest lifetime yet documented for NO in an intact tissue and is 1–2 orders of magnitude below previous theoretical guesses (Wood and Garthwaite, 1994). Unfortunately, existing methods for direct detection of NO are too slow and invasive to provide an independent test of this estimate. A lifetime of <50 ms would predict (Wood and Garthwaite, 1994) a space constant for radial decay of <13 μm, even for such a small and diffusible molecule as NO, which would be consistent with quite high but not necessarily perfect synapse specificity of LTD. The short lifetime and coincidence requirement would also provide a biochemical explanation of the physiological requirement for PF and climbing fiber activity to be synchronized. The actual spatial spread of NO action and the molecular mechanism by which coincident NO and $[Ca^{2+}]_i$ are detected are major questions for future experiments.

Experimental Procedures

Thin (200–300 μm thick) sagittal slices were cut with a Microslicer DSK-3000W (Dosaka EM Co., Japan) from the cerebellar vermis of rats aged 18–26 days. Whole-cell patch-clamp recording methods (Edwards et al., 1989; Hamill et al., 1981) were used to record voltage and current signals produced by synaptic activation in PCs. The cells were directly visualized through a 10× water immersion objective on an upright microscope (Axioplan, Carl Zeiss, Inc.). Surfaces of Purkinje somata were gently cleaned using large-bore micropipettes. Tight-seal whole-cell recordings (seal resistance > 10 GΩ) were made with patch pipettes with 3–4 MΩ resistance and an Axopatch 200A (Axon Instruments) amplifier at a holding potential of −70 mV. The intracellular solution contained 130 mM K-gluconate, 10 mM KCl, 10 mM K-HEPES, 1 mM $MgCl_2$, 4 mM Na-ATP, 1 mM Na-GTP, and 16.17 mM sucrose (pH 7.2; 300 mosm). Although these conditions appear to be adequate for measuring EPSCs, the voltage clamp is incapable of maintaining space-clamp conditions in the dendrites and axon when spikes are generated. Better space clamp, tighter voltage control, and more accurate quantitation of synaptic currents would have been obtainable in cells from younger animals (Llano et al., 1991), but because such quantitation is not essential for the findings presented here, we chose a more adult stage of development in which the dendritic arbor and LTD phenomenon are more mature. The external Ringer's solution contained 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 25 mM glucose (pH 7.4), and 10 μM (-)-bicuculline methiodide (Research Biochemical International) to inhibit GABAergic synapses. Slices were continually perfused with Ringer's solution saturated with 95% O_2, 5% CO_2. All experiments were performed at room temperature (near 22°C). Recordings of synaptic currents were considered only if they passed the following criteria: responses to low frequency (0.2 Hz) PF stimulation were constant in amplitude and time course during the initial 5–10 min of recording, and series conductance between the pipette and the PC cytoplasm remained constant within 20%.

The caged NO compound CNO-4 was previously synthesized in this laboratory (Makings and Tsien, 1994). It is indexed in *Chemical Abstracts* as acetic acid, 2,2′-[[4-[[(3,3-diethyl-1-triazenyl)oxy]methyl]-5-nitro-1,2-phenylene]bis(oxy)]bis-, N-oxide, dipotassium salt, registry number 154751-51-6. CNO-4 was introduced into the Purkinje neuron by inclusion at 200 μM in the patch pipette intracellular solution. The photolytic illumination was provided by a 200 W DC mercury arc lamp with an electromechanical shutter (Vincent Associates, Rochester, NY), mounted separately from the main body of the microscope to minimize electrical and vibrational interference. UV wavelengths, especially the prominent mercury line at 365 nm, were selected by a glass filter with peak transmission at 361 nm and half-maximal cutoffs at 316 and 388 nm. Initially, the UV light was focused onto a quartz fiber light guide and introduced through a reversed 10× objective (Edmund Scientific) into the trinocular head of the microscope and an Optovar magnification changer. This setup (dim UV) was adequate for early experiments such as those in Figures 1–3, but much intensity was wasted by the fiber and the poor UV transmittance of the reversed objective and trinocular head. As a result, long (e.g., 900 ms) exposure were necessary to photolyze sufficient CNO-4. To raise the inten-

sity so that shorter (e.g., 200 ms) exposures could be used to explore coincidence requirements, the fiber optic was eliminated and the collimated output of the lamp was aimed directly into the epifluorescence port (bright UV), with an air gap of several inches to prevent transmission of shutter vibration. Calibrations with a silicon photodiode photometer (IL1700, International Light, Newburyport, MA) indicated that dim and bright UV corresponded to 16 and 152 mW/cm^2, respectively, at the plane of focus in the absence of attenuation by overlying tissue. Based on the known extinction coefficient and quantum yield of CNO-4 (Makings and Tsien, 1994), dim and bright UV should have released 1.3 and 2.8 μM NO, respectively, per 900 ms versus 200 ms irradiation period. The actual amount of NO released in cells would probably have been smaller due to tissue opacity to UV light and any incompleteness of equilibration of the 200 μM CNO-4 from patch pipette into the neuron.

Oxymyoglobin was prepared by dissolving 5 mM myoglobin (Calbiochem) in Ringer's solution, adding 20 mM sodium dithionite to reduce the metmyoglobin to deoxymyoglobin, and separating the protein from excess dithionite by Sephadex G-25 gel filtration, during which the deoxymyoglobin reoxidized to oxymyoglobin. The concentration of repurified MbO_2 was evaluated from its absorption at 416 and 543 nm. The solution was kept at 4°C and used within 3 days.

For global NOS inhibition, slices were incubated in 50 or 100 μM N^G-nitro-L-arginine (Sigma) for 1–4 hr. For local inhibition, the NOS inhibitor was introduced into individual PCs by inclusion in the patch pipette at various concentrations (50, 10, 5, and 3 μM).

$[Ca^{2+}]_i$ increases were achieved by 50 ms step depolarizations to a voltage at which regenerative Ca^{2+} spikes were induced in the out of clamp dendritic region (Figure 1, bottom left). This voltage was individually determined for each PC at the beginning of whole-cell recording. PF stimulation was achieved using a bipolar electrode placed at the pial surface above and up to 200 μm on either side of the recorded PC. The timing of depolarization, PF stimulation, and shutter opening was orchestrated using a multichannel stimulator (Master-8, A. M. P. I., Israel). Voltage and current data were digitized and stored on a VCR recorder via a PCM2 A/D VCR adaptor (Medical System Corp., Greenvale, NY) and transferred via a PCMI2 interface to a personal computer for software extraction of synaptic current amplitudes.

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