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Synergies and Coincidence Requirements between NO, cGMP, and Ca^{2+} in the Induction of Cerebellar Long-Term Depression

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Summary

Parallel fiber synapses onto Purkinje neurons in acute cerebellar slices undergo long-term depression (LTD) when presynaptic activity coincides with postsynaptic depolarization. These electrical inputs can be respectively replaced by nitric oxide (NO) and Ca^{2+} photolytically released inside the Purkinje neuron, showing that these two messengers are sufficient for LTD induction. NO acts via cGMP production because inhibitors of guanylate cyclase prevent LTD but can be circumvented by photoreleased cGMP combined with Ca^{2+} elevation. Three inhibitors of cGMP-dependent protein kinase, *R*p-8Br-PET-cGMPS, KT5823, and a novel pseudosubstrate peptide, all block LTD. LTD induction permits $<$10 ms gap between NO release and Ca^{2+} elevation, whereas 200–300 ms is allowed between uncaged cGMP and Ca^{2+} increase. This surprising difference in timing precision can be explained either by tighter localization and faster decay of cGMP when generated by NO rather than uncaging, or by two independent coincidence detectors in series.

Introduction

Long-term depression (LTD) in the cerebellum is a form of synaptic plasticity in which a brief burst of simultaneous stimulation of the climbing fiber (CF) and parallel fiber (PF) synapses onto Purkinje neurons causes a long-lasting depression in the efficacy of the PF synapse (Ito et al., 1982; Sakurai, 1987). The CF serves merely to depolarize the Purkinje neuron and can be replaced by depolarization, which opens voltage-operated Ca^{2+} channels and produces a transient rise in intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$). This $[Ca^{2+}]_i$ transient is necessary for LTD induction because buffering of intracellular Ca^{2+}, for example with BAPTA, prevents LTD induction (Konnerth et al., 1992). However, CF stimulation or depolarization also evokes currents of sodium and other ions. Na^+ influx has been suggested to be an essential component of LTD induction in cultured Purkinje neurons derived from immature animals (Linden et al., 1993). Other workers have found that uncaging of Ca^{2+} inside similar cultured Purkinje neurons is sufficient to replace depolarization and synergizes with iontophoretic pulses of glutamate (Kasono and Hirano, 1994) to elicit LTD. Thus, although there is unanimity that Ca^{2+} is necessary for LTD induction, it remains controversial whether Ca^{2+} can completely replace depolarization in Purkinje cells (PCs) cultured for long periods from fetal animals. The same question has not been tested in mature cells in acute slices.

The participation of nitric oxide (NO) in LTD induction was first proposed by Ito and Karachot (1990), Crépel and Jaillard (1990), and Shibuki and Okada (1991), though its sources and targets remained controversial. It is now clear that NO arises from and mimics parallel fiber stimulation and is synthesized outside the PC while its target(s) is inside the PC (Lev-Ram et al., 1995). This is in agreement with the cellular localization of nitric oxide synthase (NOS) in the cerebellum (Bredt et al., 1990). The enzyme is found in granule, basket, and Bergman glia cells but not in cerebellar PCs. NO activates soluble guanylate cyclase (sGC) by binding to the heme portion of the enzyme (see Stone and Marletta [1996] and references therein). The resulting production of cGMP could activate cGMP-dependent protein kinase (PKG), though a role for cyclic nucleotide-gated cation channels is also conceivable. Another open question is what other biochemical signals must be provided along with cGMP to cause LTD. Hartell (1994a) found that microinjected 8-Br-cGMP required simultaneous PF activity to elicit LTD, but the reason for needing electrical activity remains obscure. Further confusion over the roles for NO and cGMP arise from multiple observations that these messengers are not involved in depression of glutamate-evoked currents in PCs cultured for long periods from embryonic animals (Linden and Connor, 1992).

Metabotropic glutamate receptor activation is clearly important in LTD, because pharmacological blockade of such receptors or genetic deletion of the mGluR1 subtype prevents LTD (Hartell, 1994b; Aiba et al., 1994). However, the position of mGluR1 activation in the signal transduction cascade is uncertain. Most workers have assumed that the relevant mGluR1 molecules are on the PC (Ito and Karachot, 1990; Shigemoto et al., 1992) and that their function is to cause phosphoinositide hydrolysis to release $Ins(1,4,5)P_3$ and diacylglycerol in that cell. $Ins(1,4,5)P_3$ could synergize with Ca^{2+} from depolarization to produce further release of Ca^{2+} through the $Ins(1,4,5)P_3$ receptor (Berridge, 1993), while diacylglycerol could synergize with Ca^{2+} to activate protein kinase C. It remains unclear how this potential signaling cascade relates to that of NO and cGMP. Hartell (1996) recently reported that the mGluR antagonist MCPG prevents a form of LTD produced by inhibitors of cGMP phosphodiesterase or injections of 8-Br-cGMP, which would imply that mGluRs are an essential component downstream of cGMP.

To sort out the relative importance and ordering of these molecules and pathways, we feel that it is important to go beyond traditional pharmacological activators and inhibitors. Our approach relies heavily on caged messengers (Adams and Tsien, 1993), molecules that are biologically inert until photolyzed by a flash of ultraviolet (UV) light, whereupon they immediately release the active messenger such as NO, Ca^{2+}, or cGMP. The resulting pulsed biochemical signals are more realistic

than static application of NO donors, Ca^{2+} ionophores, or nonhydrolyzable cGMP analogs. The ability of an uncaged messenger to circumvent pharmacological blockade proves specificity of the inhibitors and establishes the ordering of steps in a signal transduction cascade. The precise temporal control enables exploration of coincidence timing requirements, which are crucial in controlling when a synapse undergoes plasticity.

Results

Simultaneous PF Stimulation and Uncaging of Ca^{2+} Induce LTD

Although Ca^{2+} is necessary for LTD induction, it has been unclear whether it is also entirely sufficient to mediate the effects of depolarization in mature PCs. The simplest way to answer this question is to elevate $[Ca^{2+}]_i$

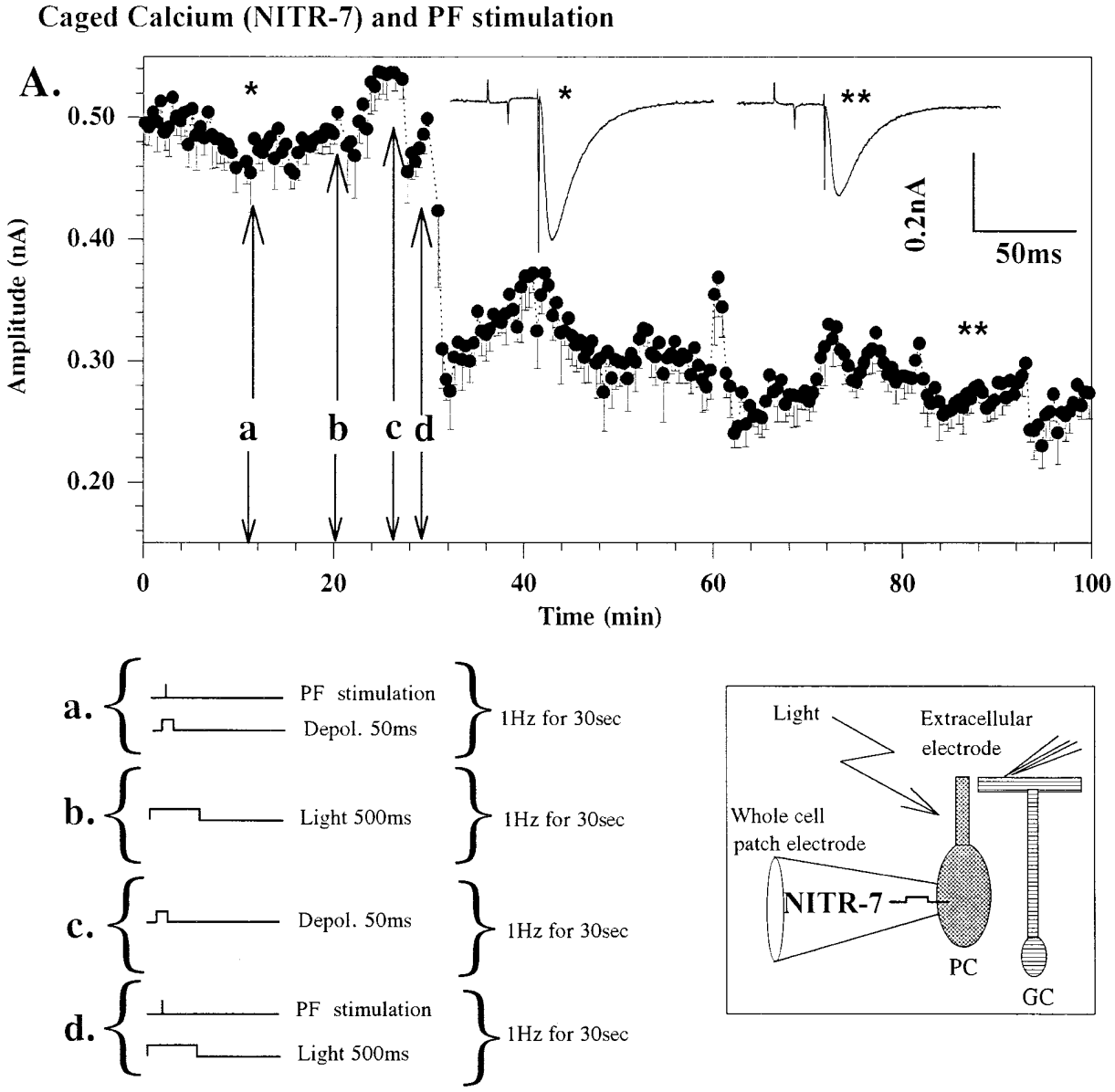

Figure 1. Induction of Cerebellar LTD by Uncaged Ca^{2+} in Conjunction with PF Stimulation

EPSCs in a Purkinje neuron are monitored using whole-cell patch recording. Each point is the average of five EPSC peak amplitudes from consecutive PF stimuli delivered at 0.2 Hz, a test frequency too low to cause plasticity. Error bars represent the SDs of each group of five responses. The patch pipette included 500 μM Ca^{2+}-free nitr-7. Simultaneous PF stimulation and depolarization at 1 Hz for 30 s, a standard training protocol (a) for eliciting LTD, was ineffective because unphotolyzed nitr-7 buffered the $[Ca^{2+}]_i$ elevation. Photolytic release of Ca^{2+} by 500 ms flashes of 365 nm light (1 Hz for 30 s) (protocol b) likewise did not change the EPSC amplitude, indicating that the UV and $[Ca^{2+}]_i$ elevation by themselves had no detectable effect. During protocol (c), nitr-7 was reloaded with Ca^{2+} from depolarization-induced $[Ca^{2+}]_i$ transients. Finally, protocol (d), in which photorelease of nitr-7 coincided with PF stimulation (1 Hz for 30 s), induced LTD, i.e., a lasting reduction in EPSC amplitude. Similar results were obtained in all of three experiments. Insets show typical averages of 10 consecutive EPSC traces from the times indicated by the single and double asterisks.

Table 1. Summary of Protocols to Determine Necessary and Sufficient Conditions for LTD Induction

Manipulation	mean %	±SE	(n)	*P*
PF + depol.	84.5	±5.73	(15)	6×10^{-3}
Uncaged NO + depol.	61.9	±5.99	(14)	7×10^{-5}
Nitr-7 + (PF and depol.), no UV	95.9	±4.55	(3)	0.22, n.s.
Ca^{2+} uncaged from nitr-7 + PF	55.8	±4.08	(3)	6×10^{-3}
Nitr-7 and caged NO + (PF and depol.), no UV	99.1	±2.36	(6)	0.20, n.s.
Uncaged (Ca^{2+} + NO)	50.1	±5.96	(7)	5×10^{-5}
ODQ + (PF and depol.)	95.6	±5.84	(6)	0.14, n.s.
ODQ + (uncaged NO and depol.)	100.8	±5.99	(5)	no depress
ODQ + (uncaged cGMP and depol.)	64.6	±3.34	(6)	5×10^{-5}
MCPG + (PF and depol.)	97.2	±4.09	(3)	0.22, n.s.
MCPG + (uncaged NO and depol.)	53.7	±14.7	(3)	1.3×10^{-2}
Rp-8Br-Pet-cGMPS + (uncaged NO and depol.)	136.9	±9.81	(13)	no depress
KT5823 + (PF and depol.)	99.8	±2.10	(7)	0.47, n.s.
KT5823 + (uncaged NO and depol.)	102.2	±3.94	(7)	no depress
PKG inhibitory peptide + (PF and depol.)	129.0	±22.8	(8)	no depress
PKG inhibitory peptide + (uncaged NO and depol.)	150.1	±35.8	(8)	no depress
cGMP uncaged from DMNB-cGMP + depol.	62.8	±3.39	(12)	1×10^{-9}
cGMP uncaged from CM-cGMP + depol.	57.8	±5.49	(13)	2×10^{-6}
cGMP uncaged from DMNB-cGMP + BAPTA + depol.	103.9	±15.1	(5)	no depress

''Mean%'' is the average EPSC amplitude measured every 5 s from 5–10 min after the manipulation, expressed as a percentage of the average EPSC amplitude for the last 5 min before the manipulation, then averaged over the number of experiments (n). ''±SE'' is the standard error of the final mean of percentages. *P* is the probability that the observed depression of mean % below 100% could have been obtained by chance, calculated by the one-tailed paired Student's *t* test (Sheskin, 1997). Values >0.05 are noted as n.s., not significant. ''No depress'' indicates that the mean after the manipulation exceeded that before the manipulation, nullifying the hypothesis that LTD was induced. PF, parallel fiber stimulation. Depol., depolarization. All protocols considered to induce LTD have $P \leq 0.013$, whereas all protocols in which LTD induction was considered blocked have $P \geq 0.14$.

by a nonelectrical means, namely photorelease of caged calcium introduced into the cell via the patch pipette. We chose nitr-7 because it has a high prephotolysis Ca^{2+} affinity, selectivity against Mg^{2+} interference, fast kinetics, and lack of proton uptake or release (Adams et al., 1988). Before photolysis, nitr-7 is a good buffer of Ca^{2+} and should mimic BAPTA in preventing LTD induction. This prediction is confirmed in Figure 1, protocol a, in which nitr-7 prevented the usual induction of LTD by PF stimulation and simultaneous depolarization (n = 3; see also Table 1). Also, when the nitr-7 was photolyzed by UV pulses (500 ms) at 1 Hz for 30 s without PF stimulation (protocol b), LTD was not induced (n = 4). However, when the uncaging was synchronized with PF stimulation (protocol d), LTD promptly resulted (n = 3). The specific need for parallel fiber stimulation indicated that the reduction in synaptic current was not due to nonspecific photodynamic damage or Ca^{2+}-induced toxicity to the participating cellular elements. Thus, photoreleased Ca^{2+} is sufficient to replace CF activity and depolarization, and the elevation of $[Ca^{2+}]_i$ is the only essential function of depolarization in inducing LTD in mature PCs in slices.

Simultaneous Uncaging of NO and Ca^{2+} Induce LTD without Any Electrical Activity

We have previously shown that PF stimulation can be entirely replaced by uncaging of NO (Lev-Ram et al., 1995). Therefore, simultaneous photorelease of NO and Ca^{2+} should be sufficient to induce LTD. Figure 2 confirms this prediction. The two caged compounds, CNO-4 and nitr-7, were both introduced into the PC via the patch pipette. Simultaneous depolarization and PF activity failed to induce LTD (protocol a; n = 6) just as in Figure 1, presumably because of the Ca^{2+}-buffering action of the unphotolyzed nitr-7. This stimulation also served to make sure that the nitr-7 was loaded with Ca^{2+}. Then UV illumination to release both NO and Ca^{2+}, without any electrical or synaptic stimulation (protocol b; n = 7), produced LTD. This result was obtained in all of seven cells tested and strongly indicates that NO and Ca^{2+} together are sufficient intracellular triggers for all subsequent biochemistry.

Coincidence Timing Requirements for NO and Depolarization

Caged compounds are uniquely advantageous for determining biochemical timing requirements since they deliver messenger substances with temporal precision. CNO-4 belongs to a family of molecules whose release of NO tracks the time course of illumination with <5 ms delay (Makings and Tsien, 1994). Such fast kinetics permit exploration of the coincidence requirement for NO and depolarization to induce LTD. However, we could not combine caged Ca^{2+} and caged NO to explore timing delays because they are photolyzed by the same UV wavelengths. Therefore, we returned to depolarization as a rapid means for elevating $[Ca^{2+}]_i$ and tested how much time delay is tolerated between the end of NO uncaging and the subsequent onset of depolarization. Previously, we had shown that 50 ms delay was sufficient to thwart LTD, but the actual threshold was not determined (Lev-Ram et al., 1995). Figure 3 shows an example in which 10 ms is borderline, giving about one third the maximal degree of synaptic depression that coincident NO and depolarization could produce (Figure 3A, protocols a and b). On average (Figure 3C), 10 ms delay gives 50% of maximal LTD (n = 4), and delays of 50 ms or greater prevent it altogether.

Both in vivo and in slices, LTD has long been known to require near synchrony between the CF and PF inputs (Ekerot and Kano, 1989; Schreurs and Alkon, 1993).

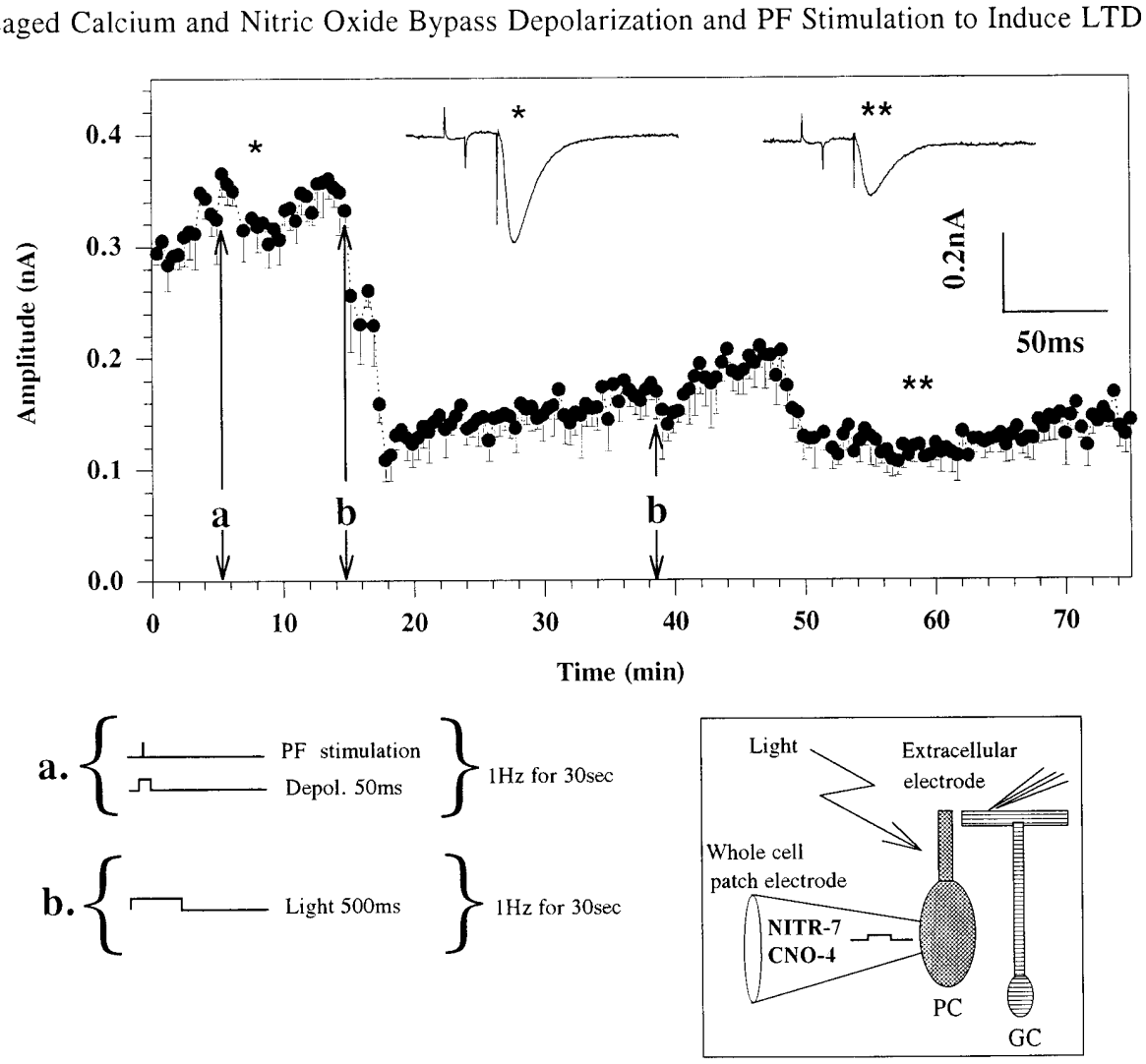

Figure 2. Induction of Cerebellar LTD by Simultaneously Photoreleased Ca^{2+} and NO

The patch pipette included caged calcium (500 μM nitr-7) and caged NO (200 μM CNO-4 dipotassium salt). LTD induction by the standard protocol (a) of PF stimulation and depolarization (1 Hz for 30 s) failed as in Figure 1 because nitr-7 buffered $[Ca^{2+}]_i$. However, simultaneously photoreleased NO and Ca^{2+} (protocol b, 500 ms UV, 1 Hz, 30 s), without any depolarization or presynaptic stimulation, resulted in LTD. A second application of protocol (b) had no further effect, indicating that the initial effect was saturable and arguing against any progressive photodynamic damage. Similar results were obtained in all of seven experiments. Insets show typical averages of 10 consecutive EPSC traces from the times indicated by the single and double asterisks.

However, the estimates of timing resolution varied considerably and had not been measured under our present conditions in slices with depolarization supplied by a whole-cell patch pipet. We now find that PF stimulation preceding depolarization by just 20 ms is completely ineffective and may even produce some potentiation, while a delay of 10 ms produced incomplete LTD, and the two inputs simultaneously give full LTD (Figures 3B and 3C). Thus, PF activity and uncaged NO are very similar in their precision of coincidence detection with depolarization-induced Ca^{2+} elevation.

LTD Produced by Uncaged NO + Depolarization Is Independent of mGluR Activation

Our previous results that Ca^{2+}, either from depolarization (Lev-Ram et al., 1995) or nitr-7 photolysis (Figure 2), synergizes with uncaged NO to induce LTD without any presynaptic activity or transmitter release suggest that subsequent mGluR activation is unnecessary. However, Hartell (1996) challenged this conclusion because he found that the mGluR inhibitor MCPG (*RS*-α-methyl-4-carboxyphenylglycine) prevented LTD induced by PF activity plus either phosphodiesterase inhibitors or injection of 8-Br-cGMP. We therefore tested MCPG explicitly. Application of 200 μM MCPG in the bath prevents LTD induction by PF stimulation plus depolarization (Figure 4, protocol a) as expected (Hartell, 1994b, 1996; Hémart et al., 1995) but does not block the effect of uncaged NO plus depolarization (Figure 4, protocol b; n = 3). This result confirms that NO + Ca^{2+} elevations together in the PC bypass mGluRs and suggests that the crucial mGluRs act upstream of endoge-

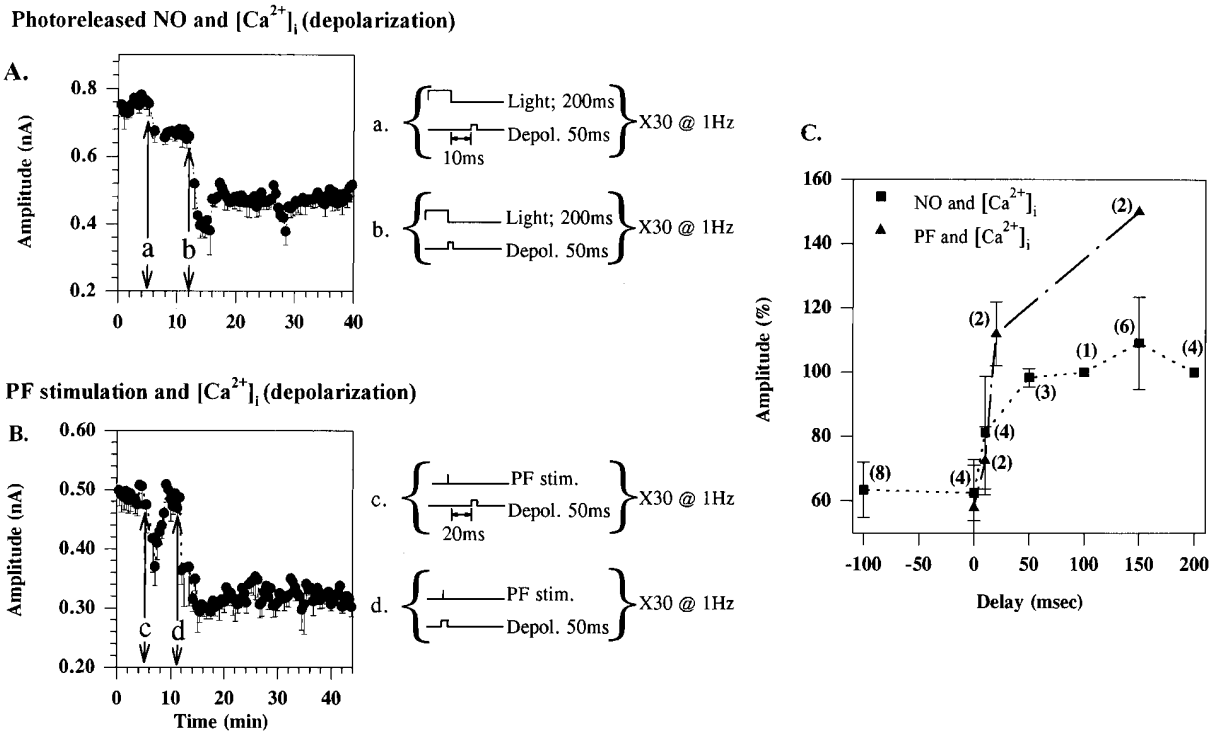

Figure 3. LTD Induction Requires <~10 ms Delay between Cessation of NO Release and Onset of Depolarization

(A) The patch pipette contained 200 μM CNO-4 in addition to the standard intracellular buffer. Coincidence detection between NO and Ca^{2+} was investigated by delaying the onset of the 50 ms depolarization by variable intervals after the end of the 200 ms uncaging illumination. A typical experiment with a 10 ms delay (protocol a) caused partial LTD, but the same stimuli with no gap (protocol b) induced full LTD. Due to the finite speed of the electromechanical shutter and of CNO-4 photolysis kinetics (Makings and Tsien, 1994), NO release may have continued for up to 5 ms after the end of the shutter command pulse. However, a comparable delay of ~5 ms occurs from the onset of depolarization to the time when the $[Ca^{2+}]_i$ rise reaches half-maximal amplitude (Miyakawa et al., 1992). These two delays should roughly cancel when calculating the actual delay between removing NO production and supplying Ca^{2+}.

(B) The patch pipette contained the standard intracellular buffer. Coincidence detection between PF stimulation and Ca^{2+} was investigated by delaying the onset of the 50 ms depolarization by variable intervals after the PF stimulation. A typical experiment in which the stimuli separated by a 20 ms gap (protocol c) caused a transient but no sustained depression, but the same stimuli with no gap (protocol d) induced full LTD.

(C) Summary of the mean posttraining synaptic amplitudes as percentages of pretraining baseline for all delays tested (photoreleased NO—closed squares; PF stimulation—closed triangles). Numbers of trials are indicated in parentheses next to each point. A delay of −100 ms indicates depolarization within rather than after the 200 ms illumination pulse.

nous NO generation. Because such NO comes not from the PC but from the parallel fibers or associated cells like basket or Bergman glia (Lev-Ram et al., 1995), mGluRs on those cells may play an essential role in promoting NOS activity. Glaum et al. (1992) have shown that presynaptic mGlu autoreceptors as well as the better known postsynaptic mGluRs are present at the PF–PC synapse.

Inhibition of Soluble Guanylate Cyclase Prevents LTD Induction, but Uncaged cGMP Can Circumvent the Blockade

The best-defined molecular target of NO is sGC (Bredt and Snyder, 1992). NO stimulates sGC to generate cGMP (Bredt and Snyder, 1989; Furchgott and Vanhoutte, 1989), which in turn activates cGMP-dependent protein kinase (PKG) (Ito, 1991; Ito and Karachot, 1992). Moreover, this is a likely pathway since sGC is abundant in PCs (Nakane et al., 1983; Matsuoka et al., 1992), and there is already considerable evidence for the involvement of cGMP in LTD. If cGMP is downstream of NO, inhibition of sGC should prevent induction of LTD by either the PF or uncaged NO, but uncaged cGMP should bypass this blockade. These predictions are confirmed in Figure 5A, showing the effects of a new NO-stimulated guanylate cyclase inhibitor, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]-quinoxalin-1-one (ODQ), added to the bath solution. ODQ is the most specific blocker currently available for sGC, with an IC_{50} of 20 nM (Garthwaite et al., 1995). As predicted, 1 μM ODQ prevents either PF stimulation (protocol a; n = 6) or photoreleased NO (protocol b; n = 5) from inducing LTD. This blockade can be circumvented by uncaged cGMP plus depolarization (Figure 5B; n = 6). Another guanylate cyclase inhibitor, LY83583 (Schmidt et al., 1985), bath applied at 1 μM, also blocked the effects of PF stimulation or photoreleased NO (results not shown), though these results are less definitive because LY83583 is less potent and probably less specific than ODQ. These results suggest that NO acts by elevating cGMP in the Purkinje cell.

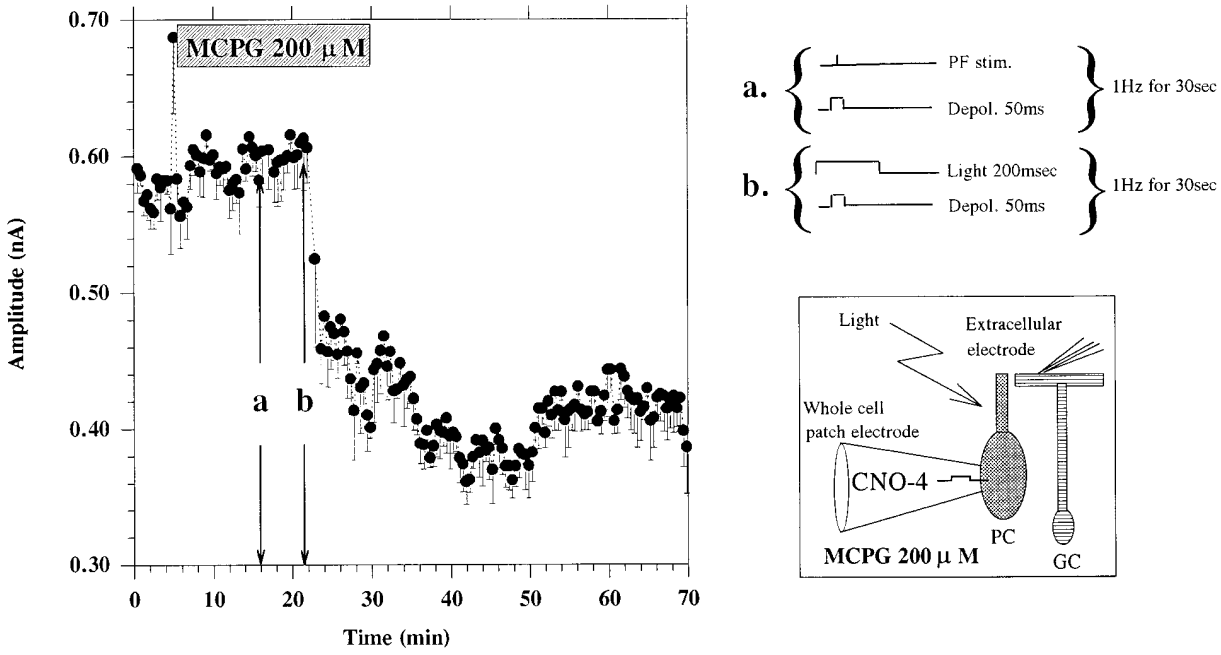

Figure 4. Blockade of Metabotropic Glutamate Receptors Can Be Circumvented by Photoreleased NO Plus Ca^{2+}

MCPG, a general antagonist of mGluRs, was included at 200 μM in the bathing solution during the period indicated. A small artifact accompanied the onset of MCPG perfusion. MCPG prevented LTD induction by PF stimulation and depolarization (protocol a). However, NO (photoreleased from 200 μM CNO-4) and depolarization (protocol b) circumvented the blockade in all of three experiments.

Inhibition of cGMP-Dependent Protein Kinase Prevents LTD Induction

Further evidence that cGMP is essential for LTD and acts through protein phosphorylation comes from experiments with three different inhibitors of PKG. R_P-8-Br-PET-cGMPS is an inhibitor of PKG type 1a and b with K_i values of 35 nM and 30 nM, respectively, and is not metabolized by mammalian cyclic nucleotide phosphodiesterase (PDE) (Butt et al., 1995). This cGMP analog, which presumably competes for the cGMP binding site, was introduced into the PC via the patch pipette (500 nM) and prevented LTD induction by either PF stimulation or photoreleased NO, both combined with depolarization in all cells tested (Figure 6A). Another way to block PKG is by KT5823, a relatively specific PKG inhibitor (Grider, 1993) that competes for the ATP binding site on the kinase (Kase et al., 1987). Application of KT5823 at 1 μM in the bath did not change the electrophysiological properties of the cell. However, it prevented LTD induction by both PF stimulation or photoreleased NO, each combined with depolarization (n = 7) (Figure 6B). KT5823 had previously been reported to prevent LTD induction by PF+CF stimulation or microinjected 8-Br-cGMP (Hartell, 1994a).

A third independent way to inhibit PKG is with a pseudosubstrate peptide to block the phosphoacceptor binding site. The novel peptide Gly-Arg-Thr-Gly-Arg-Arg-Asn-(D-Ala)-Ile-NH_2 (PKGI) is the most specific PKG inhibitory pseudosubstrate currently available (J. S. W., M. S. Mendelow, and D. S. L., unpublished data). PKGI competitively inhibits phosphorylation of a model substrate, kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly-NH_2), by PKG and PKA (cAMP-dependent protein kinase). The respective K_is are 14.8 μM and 1.02 mM, a selectivity of 70 in favor of PKG. PKC is even less affected, being inhibited only 20% at 1 mM. The peptide was introduced at 1 mM in the patch pipette and was allowed to diffuse into the cell for 25 min. The intracellular presence of PKGI prevented LTD induction by either PF stimulation or photoreleased NO, both combined with depolarization (Figure 6C). Although any inhibitor might be nonspecific, the ability of three very different types of PKG blockers to prevent LTD generation is strong evidence for the necessity of this enzyme in LTD induction by PF stimulation or NO. The efficacy of KT5823 and PKGI argues strongly for PKG rather than cyclic nucleotide-gated channels to be the key target for cGMP activation.

Photoreleased cGMP Still Needs a $[Ca^{2+}]_i$ Increase to Cause LTD

Does cGMP still need Ca^{2+} to induce LTD? Uncaging cGMP by itself elicited full LTD in only three of seven trials. However, uncaged cGMP combined with depolarization-induced Ca^{2+} transients was much more reliable, eliciting LTD in 28 of 31 experiments. We hypothesized that the borderline results with uncaged cGMP alone may have been ambiguous because $[Ca^{2+}]_i$ was then insufficiently controlled. Therefore, the Ca^{2+} chelator BAPTA (5 mM) was included in the patch pipette. In five of six cells, Ca^{2+} buffering by BAPTA prevented both PF stimulation and uncaged cGMP from inducing LTD, even when depolarizations were included (Figure 7C; n = 5). Thus, even after NO has stimulated cGMP production, Ca^{2+} elevation is still required. Table 1 summarizes the statistics of the protocols that succeeded or failed to induce LTD.

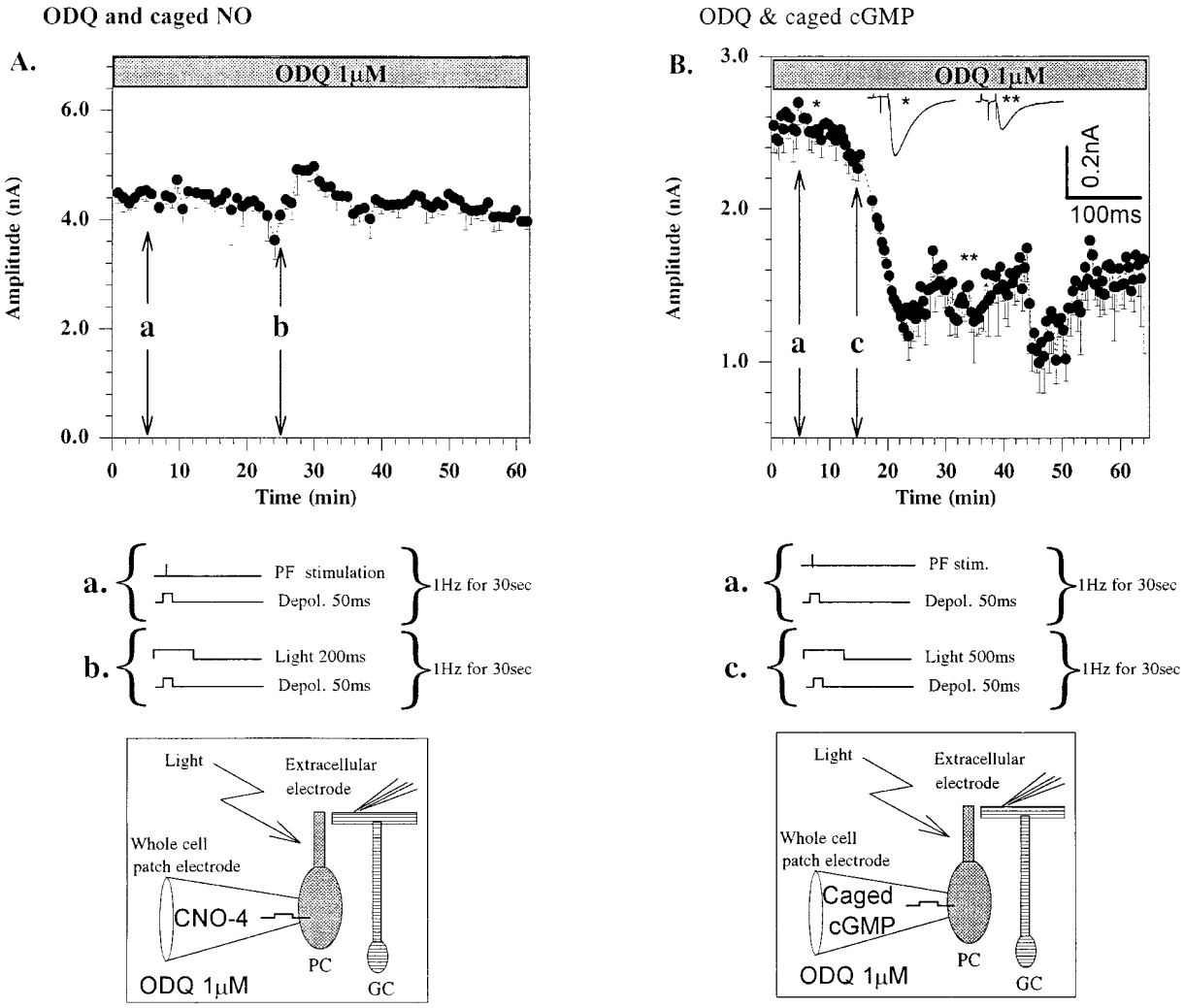

Figure 5. ODQ Prevents LTD Resulting from PF Activity or Uncaged NO but Can Be Circumvented by Uncaged cGMP Plus Ca^{2+}

(A) With 1 μM ODQ in the bath to inhibit NO-stimulated sGC, PF stimulation (protocol a) or NO uncaging (protocol b), both in conjuction with depolarization, failed to produce LTD (n = 6 and n = 5, respectively).

(B) After protocol a failed to give an effect, uncaging of cGMP (500 ms UV pulses at 1 Hz for 30 s) from 187 μM NPE-cGMP in the pipette, combined with simultaneous depolarization (protocol c), circumvented the blockade of sGC and induced LTD. This experiment was typical of the six of eight trials in which uncaged cGMP and depolarization induced LTD despite ODQ blockade. Insets show typical averages of 10 consecutive EPSC traces from the times indicated by the single and double asterisks.

Uncaged cGMP Remains Effective with Up to 200–300 ms Delay before $[Ca^{2+}]_i$ Elevation

How precisely must cGMP and $[Ca^{2+}]_i$ elevations coincide? This obvious next question can be answered by inserting variable time delays between cGMP uncaging and subsequent depolarization. Figures 7 and 8A reveal that LTD is still ~50% of maximal at time delays of 300 ms, in contrast to the ~10 ms coincidence requirement for NO and $[Ca^{2+}]_i$ (Figure 3). Note that the 300 ms persistence of cGMP effectiveness is not due to uncaging a vast excess of cGMP. Reduction in the amount of light by a factor of two eliminated LTD, so the cGMP dose was <2-fold over threshold.

Could NO have a dual effect, stimulating both cGMP synthesis and breakdown, therefore narrowing the time window during which Ca^{2+} could synergize with cGMP? This hypothesis would predict that simultaneously uncaged NO and cGMP should require precise coincidence with depolarization, like NO alone rather than cGMP alone. However, Figure 8B shows that NO + cGMP act like cGMP alone, i.e., that NO cannot restrict the timing tolerance of cGMP.

Another possible artifact was that the caged cGMPs up to now were the standard 2-nitrophenethyl or 4, 5-dimethoxy-2-nitrobenzyl esters (NPE-cGMP or DMNB-cGMP), which have no net charge and are at least somewhat membrane permeant (Nerbonne et al., 1984). Because the caged cGMP is delivered through the patch pipette on the cell body, it perhaps remains unevenly distributed within the cell, with the highest concentration at the cell body. In the dendrites and postsynaptic spines, the concentration of NPE- or DMNB-cGMP

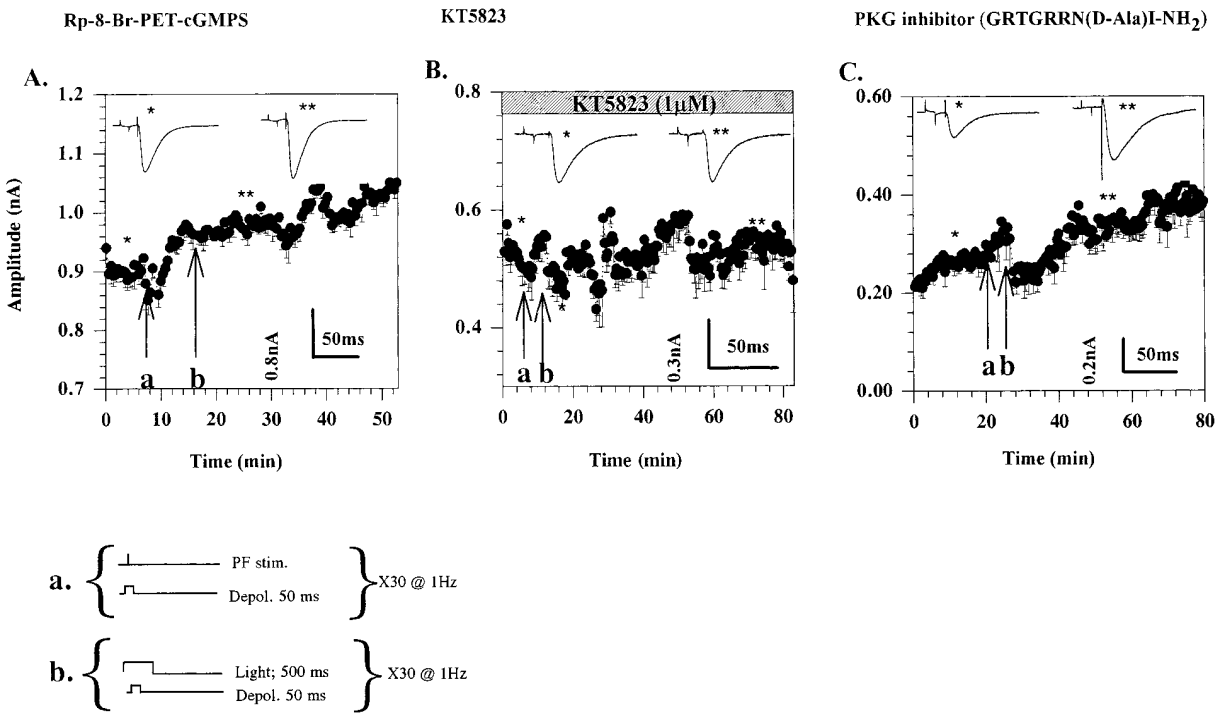

Figure 6. Three Different PKG Inhibitors Prevent LTD Induction

Neither PF stimulation nor NO photoreleased from 200 μM CNO-4 in the pipette, both combined with depolarization (protocols a and b, respectively), could elicit LTD in the presence of the following PKG inhibitors:

(A) The nonhydrolyzable cGMP antagonist Rp-8-Br-PET-cGMPS (500 nM in the pipette, n = 13).

(B) The ATP site inhibitor KT5823 (1 μM in the bathing solution, n = 7).

(C) A pseudosubstrate peptide inhibitor, GRTGRRN(D-Ala)I-NH_2, 1 mM in the pipette, n = 8). All three PKG inhibitors caused some variable degree of potentiation.

might be much lower due to efflux across their relatively large areas of surface membrane. A spatially uniform pulse of UV light would therefore release a higher concentration of cGMP at the cell body than at the relevant peripheral synapses (Figure 9A). Some of the ~300 ms allowable between cessation of uncaging and the onset of depolarization might represent the time for centrifugal diffusion of the cGMP down its concentration gradient toward the postsynaptic elements. To assess this possible artifact, a new caged cGMP (CM-cGMP) with the exact same caging group as CNO-4 was synthesized. Its structure is shown as the inset to Figure 8C. CM-cGMP has two fixed negative charges to make it much more water soluble and membrane impermeant than NPE- or DMNB-cGMP. Because of the molecular weight and hydrophilicity of its guanine and ribose moieties, CM-cGMP should be even less membrane permeant than CNO-4, and upon photolysis, should therefore give a spatially uniform increase in cGMP (Figure 9B). Indeed, CM-cGMP induced LTD when its photoreleasing pulse was synchronized with depolarization-induced $[Ca^{2+}]_i$ transient (12 of 14 experiments). LTD was reduced by ~50% when the depolarizing pulse was delayed by 200 ms after the end of photolysis (Figure 8C). Therefore, centrifugal diffusion of cGMP might explain a third of the 300 ms delay allowed with DMNB-cGMP. Nevertheless, a large discrepancy remains between the ~200 ms tolerance of cGMP uncaged from CM-cGMP and the ~10 ms tolerance for NO uncaged from CNO-4 or delivered from PF stimulation.

Discussion

Cerebellar LTD in vivo results from PF input coincident with depolarization from the CF. Many components of the signal transduction cascade have been proposed, including mGluR activation, IP_3, Ca^{2+}, diacylglycerol, Na^+, NO, and cGMP. However, the logical relationships between these components, the synaptic inputs, and the output response have been obscure or even contradictory. Our approach to untangling the maze of interactions has been the application of a variety of caged compounds that release important messengers such as Ca^{2+}, NO, and cGMP upon photolysis. Such uncaging is a very direct means to prove what functions a given messenger is sufficient for and is particularly powerful when it can circumvent inhibition of upstream signals. In such cases, the analysis is reminiscent of genetic epistasis, where the logical order of gene products in a signal transduction cascade can be deduced by the ability of mutations in downstream genes to override the effects of upstream elements. However, caged compounds have the added feature of millisecond time resolution, which helps separate the acute direct effects of a messenger from slower indirect or feedback responses,

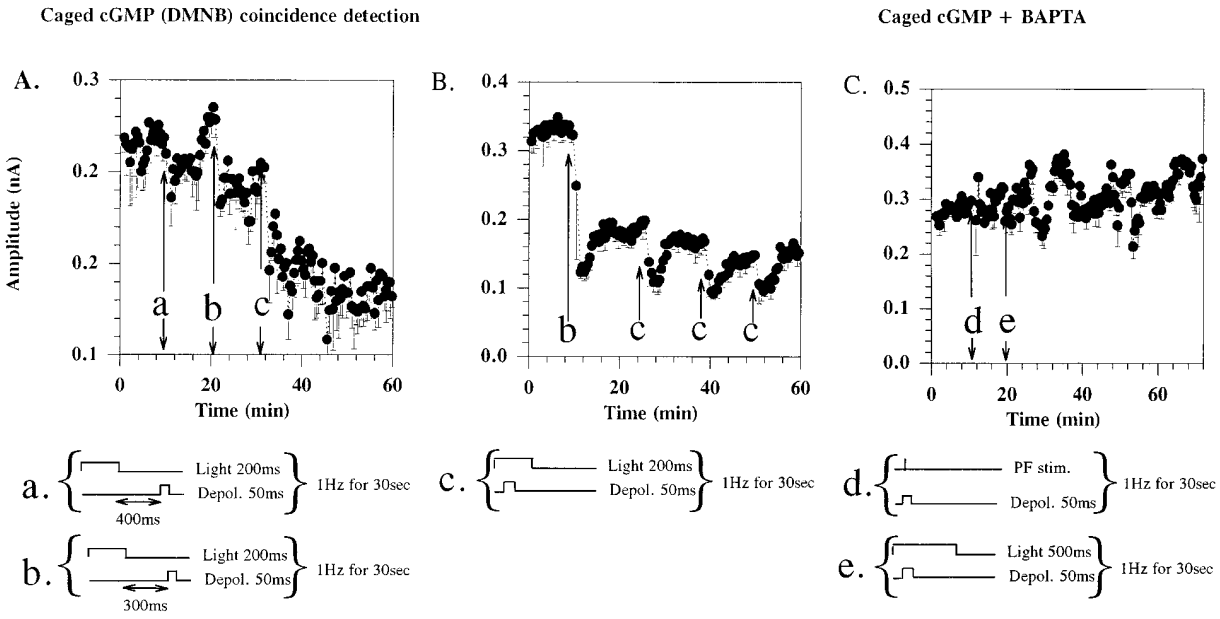

Figure 7. Uncaged cGMP Requires a Loosely Coincident $[Ca^{2+}]_i$ Transient to Produce LTD

(A and B) cGMP was uncaged by 200 ms illumination periods from 75 μM DMNB-cGMP in the pipette. When depolarization was delayed 400 ms after the end of photolysis ([A], protocol a), LTD remained absent in four of four experiments. 300 ms delay (protocol b) permitted partial (A) LTD in four of five experiments; in the remaining experiment, full LTD was observed (B). Delays of 200 ms or 100 ms resulted in yet larger reductions in EPSC amplitude, similar to overlapping depolarization and cGMP photorelease (protocol c).

(C) cGMP needs a rise in $[Ca^{2+}]_i$ to cause LTD. When NPE-cGMP (187 μM) and the Ca^{2+} chelator BAPTA (5 mM, Ca^{2+} free) were included in the pipette solution, neither PF stimulation nor uncaging with 500 ms UV could induce LTD despite concurrent depolarization. This experiment was typical of five of six experiments; in the one exception, 20% LTD was observed.

and which enables direct testing of biochemical coincidence requirements. Coincidence detection is particularly important in neural networks that have to detect significant associations between different stimulus pathways and improve their performance without an artificial teacher to revise synaptic weights (Bourne and Nicoll, 1993; Montague and Sejnowski, 1994).

We previously demonstrated that PF stimulation can be replaced by photoreleased NO (Lev-Ram et al., 1995). Either one synergizes with depolarization to elicit LTD. We have now shown that depolarization can be replaced by uncaged Ca^{2+}. To our knowledge, the combination of simultaneously uncaged Ca^{2+} and NO (Figure 2) is the first purely biochemical protocol for inducing LTD without electrical or neurotransmitter activity and is strong evidence that these two messengers are sufficient biochemical inputs. One previous report also indicated that uncaged Ca^{2+} is sufficient to synergize with iontophoretic application of glutamate to Purkinje neurons in culture (Kasono and Hirano, 1994). However, in view of the significant differences between the mechanisms of LTD in neurons cultured from immature animals verses those in slices from young adults, it was necessary to recheck this question in our preparation. Our ability to replace depolarization by photoreleased Ca^{2+} confirms that $[Ca^{2+}]_i$ elevation is the only direct essential contribution of either depolarization or CF stimulation to LTD induction in the acute slice preparation. The requirement for Na^+ elevation and the unimportance of NO and cGMP for LTD in Purkinje neurons cultured from embryonic animals (Linden and Connor, 1992; Linden et al., 1993) presumably reflect fundamental differences between that preparation and acute cerebellar slices.

We previously showed that LTD fails to occur with as little as 50 ms gap between the end of NO uncaging and the beginning of depolarization to raise $[Ca^{2+}]_i$. However, 50 ms was only an upper limit on the tolerance. Figure 3 now shows that 10 ms delay on average gives half-maximal amplitude of LTD. The clear implication is that an effective concentration of NO in the Purkinje neuron lasts for only ~10 ms after termination of NO delivery, and that NO and Ca^{2+} transients must coincide within this same time window to be effective. If NO were longer-lived or if the coincidence detection were less precise, much greater time delays between termination of NO release and commencement of Ca^{2+} elevation should have elicited LTD.

The temporal precision of NO signaling has several interesting consequences. Based on the diffusion constant of NO in free solution, 3.3×10^{-5} cm^2s^{-1} (Malinski et al., 1993), and a decay half life of 10 ms, the space constant for the concentration gradient of NO diffusing from a localized source (Wood and Garthwaite, 1994) would be only 7 μm. Contrary to common assumptions that NO is a long-range signal, NO could be local enough to explain input specificity of LTD induction. The NO–Ca^{2+} coincidence detector also explains previous findings that LTD in vivo and in slices tolerates no more than 20 ms between PF and subsequent CF activation (Ekerot and Kano, 1989; Schreurs and Alkon, 1993), which was confirmed in our patch-clamped preparation (Figure 3). A slightly greater time delay might be tolerated

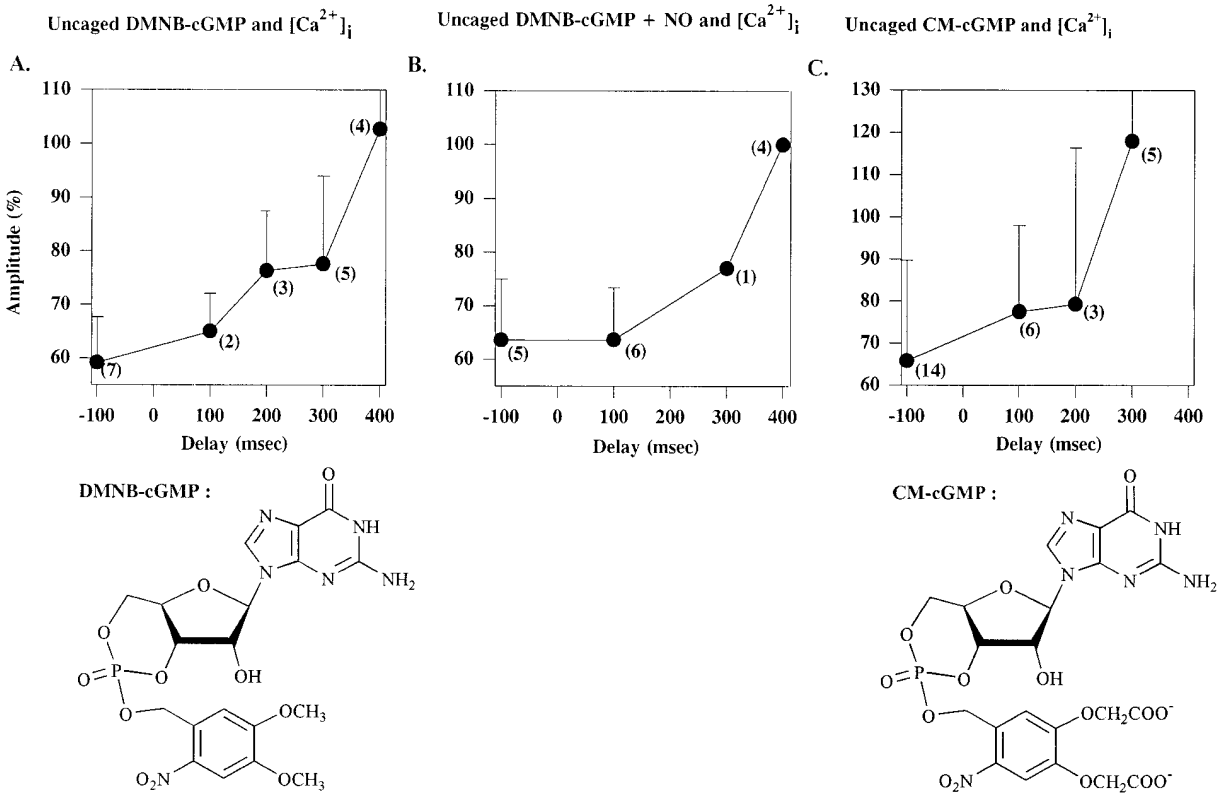

Figure 8. LTD Induction Requires <200–300 ms Delay between Cessation of cGMP Release and Onset of Depolarization

Average LTD obtained with different caging groups on the cGMP is plotted as a function of time between the end of photolysis and the beginning of Ca^{2+} elevation. Maximal LTD occurs when the depolarizing pulse is during the 200 ms of illumination (delay = −100 ms).

(A) DMNB-cGMP gives >50% of maximal LTD with up to 300 ms gap between uncaging and depolarization, whereas 400 ms does not permit LTD induction.

(B) Addition of caged NO (CNO-4, 200 μM) to the DMNB-cGMP (75 μM) in the patch pipette does not alter the timing requirements.

(C) CM-cGMP, which has two additional negative charges to prevent membrane permeation, gives partial LTD, with up to 200 ms gap between uncaging and depolarization, but fails completely at 300 ms delay. Numbers of trials are indicated in parentheses next to each point.

after PF activity compared to NO uncaging, perhaps because NO generation may continue for a few ms longer after cessation of PF activity. By contrast, the reverse order of stimulus presentation, depolarization terminating before NO release, measures the persistence of $[Ca^{2+}]_i$ elevations and permits 100–150 ms delay (Lev-Ram et al., 1995). Again, this coincidence requirement agrees with in vivo findings that up to 125 ms but not 250 ms delays between CF and PF stimulation allow LTD formation (Ekerot and Kano, 1989). Although Ca^{2+} is generally considered one of the most dynamic of intracellular messengers, NO appears to be an order of magnitude faster in this system and should be thought of as resembling a classical fast neurotransmitter.

The best-defined molecular target for NO is sGC, though many others, such as thiols in general (Hess et al., 1994), the NMDA receptor (Lipton et al., 1996), ADP-ribosyl transferase (Schuman et al., 1996), and iron response elements (Jaffrey et al., 1994), have also been invoked. Several previous reports have supported cGMP involvement in LTD in acute adult cerebellar slices (Ito and Karachot, 1990; Hartell, 1994a, 1996) if not in culture. Our previous finding that uncaged CO could substitute for NO (Lev-Ram et al., 1995) implied that guanylate cyclase is the most likely receptor because this molecule is the only known common target for both gases. Figure 5 now provides much more definitive evidence. ODQ, the most potent and specific inhibitor of sGC (Garthwaite et al., 1995), prevents NO-mediated LTD but can be bypassed by uncaging of cGMP as long as Ca^{2+} elevation is also present. The ability of cGMP to circumvent ODQ blockade clearly places NO upstream of cGMP, shows that ODQ is acting specifically, and argues that no other actions of NO are essential. Surprisingly, elevation of cGMP by uncaging is not sufficient and still requires coincident Ca^{2+} for LTD induction (Figure 7).

The requirement for some $[Ca^{2+}]_i$ elevation together with cGMP probably explains why Hartell (1994a) could only induce LTD with microinjected 8-bromo-cGMP if he also delivered 1 Hz parallel fiber stimulation. Eilers et al. (1995) have shown that such parallel fiber activity induces significant local elevations of $[Ca^{2+}]_i$, especially when no attempt is made to control membrane potential or $[Ca^{2+}]_i$, as in Hartell's cells under current clamp. In our view, much less ambiguous results are obtained by deliberately raising $[Ca^{2+}]_i$ by depolarization (Figure 5) versus clamping $[Ca^{2+}]_i$ by addition of BAPTA to the

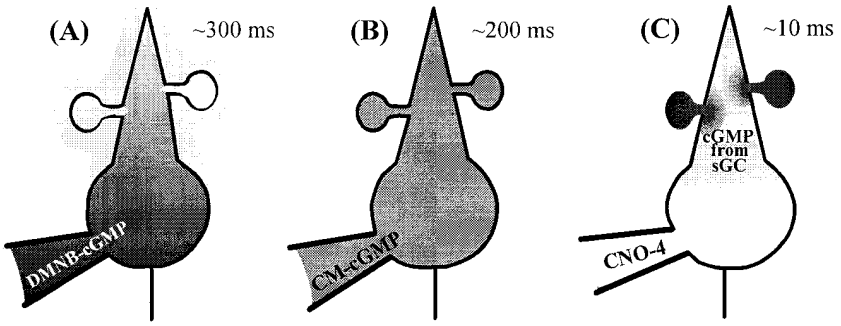

Figure 9. Possible Spatial Distributions of cGMP Generated by Different Uncaging Strategies

The highly schematized cartoons depict a Purkinje neuron with a patch-clamp electrode perfusing the cell body, an apical dendrite, and two postsynaptic spines. The shading represents cGMP levels just after a spatially uniform flash. Darker tones indicate higher cGMP concentrations.

(A) Presumed cGMP levels after uncaging of DMNB-cGMP or NPE-cGMP. Because these cGMP esters are somewhat membrane-permeant, their concentrations are likely to decline with distance from the cell body and with increasing surface-to-volume ratio as in spines. A cloud of leaked ester may even be just outside the cell. The cGMP concentration profile just after photolysis would reflect the ester concentrations before photolysis.

(B) Presumed cGMP levels after uncaging CM-cGMP. The two negative charges of CM-cGMP should greatly reduce membrane permeability and spatial nonuniformity of uncaged cGMP inside the cell. Subsequent decay of cGMP would require enzymatic hydrolysis.

(C) Presumed cGMP levels after uncaging CNO-4 to release NO, assuming that sGC is localized to the spine heads. Although the NO from the CNO-4 would be spatially rather uniform, the cGMP would rise preferentially in the spines. NO derived from normal PF activity would be preferentially located near the active synapses on spines, further accentuating the cGMP localization. In either case, once cGMP production had shut off, local cGMP levels in postsynaptic spines could fall very rapidly by simple diffusion.

internal perfusate (Figure 7). In addition, voltage clamping of the cell body as in our protocol reduces the $[Ca^{2+}]_i$ transients due to parallel fiber activity (Eilers et al., 1995).

Our most unexpected and difficult to explain finding is that LTD induction allows cGMP to precede $\Delta[Ca^{2+}]_i$ by ~200 ms (Figures 7 and 8), whereas uncaged NO must precede $\Delta[Ca^{2+}]_i$ by no more than ~10 ms (Figure 3), even though NO is upstream of cGMP (Figure 5). A single detector of coincidence between NO and Ca^{2+} cannot explain why cGMP still needs Ca^{2+}. A simple detector of coincidence between cGMP and Ca^{2+} cannot explain why the signal generated by NO is so much shorter-lived than cGMP seems to be. Various artifactual explanations such as massive overdosing of uncaged cGMP or leakage of DMNB-cGMP from the cell were tested and found to be implausible. Also, NO does not curtail the duration of action of uncaged cGMP, since simultaneous uncaging of both NO and cGMP proved no different from cGMP alone (Figure 8B).

One hypothesis is that the cGMP produced by parallel fiber activity or uncaged NO is spatially more localized (Figure 9C) and therefore much more transient than that produced by uncaging cGMP (Figures 9A and 9B). It would make sense for the sGC and PKG to be preferentially localized in the Purkinje neuron's spines together with their ultimate targets, the glutamate receptors of the AMPA subtype (Ito and Karachot, 1990; Hémart et al., 1994). Even if NO were uncaged uniformly throughout the Purkinje neuron, cGMP synthesis might be confined to the spines (Figure 9C). Upon cessation of cGMP production, the local cGMP levels could fall within milliseconds, simply by diffusion of cGMP into the much larger volume of the dendritic shafts a micron or so away. By contrast, cGMP produced by uncaging would be roughly spatially uniform throughout the Purkinje cell (Figure 9B), so the cGMP concentrations could fall only by enzymatic destruction, whose time constant could well be hundreds of milliseconds. Thus, a single fast detector of coincidence between cGMP and Ca^{2+} together with different spatiotemporal dynamics for NO-generated versus uncaged cGMP (Figure 9C) could explain the present results. This model and its supporting evidence are summarized in Figure 10A.

Experimental precedent exists for preferential generation of cyclic nucleotides at postsynaptic sites. In lobster stomatogastric neurons during stimulation of afferent synapses, direct imaging of cAMP increases reveal that they begin at the finest distal processes and subsequently propagate into thicker and more central regions of the neuron (Hempel et al., 1996). Localization of transient cGMP to spines would help explain why immunohistochemistry has been unable to detect cGMP increases in Purkinje neurons stimulated by NO donors (Southam et al., 1992; Wood et al., 1994), despite the undoubted high concentrations of sGC and PKG in these cells (Nakane et al., 1983; Matsuoka et al., 1992). It would be most interesting to image cGMP directly in live Purkinje neurons, but this would require reengineering the fluorescent sensor of cAMP to respond selectively to cGMP. This difficult task is underway in our laboratory. The hypothesis of local cGMP production also predicts that sGC is not floating free in the cytoplasm as its name may imply but rather is preferentially localized to spines. Also, cGMP accumulation would need to turn off in milliseconds after NO is withdrawn. No such biochemical mechanism is known yet perhaps because current in vitro biochemical studies of sGC, e.g., Stone and Marletta (1996), are based on the enzyme from lung, which has no need to participate in millisecond temporal discrimination. Furthermore, purified sGC is isolated from its natural environment, which contains other factors such as PKG or phosphodiesterases that might turn it off or degrade its product.

An alternative explanation is to postulate two separate coincidence detectors, one for NO and Ca^{2+}, another for cGMP and Ca^{2+} (Figure 10B). Unfortunately, the most

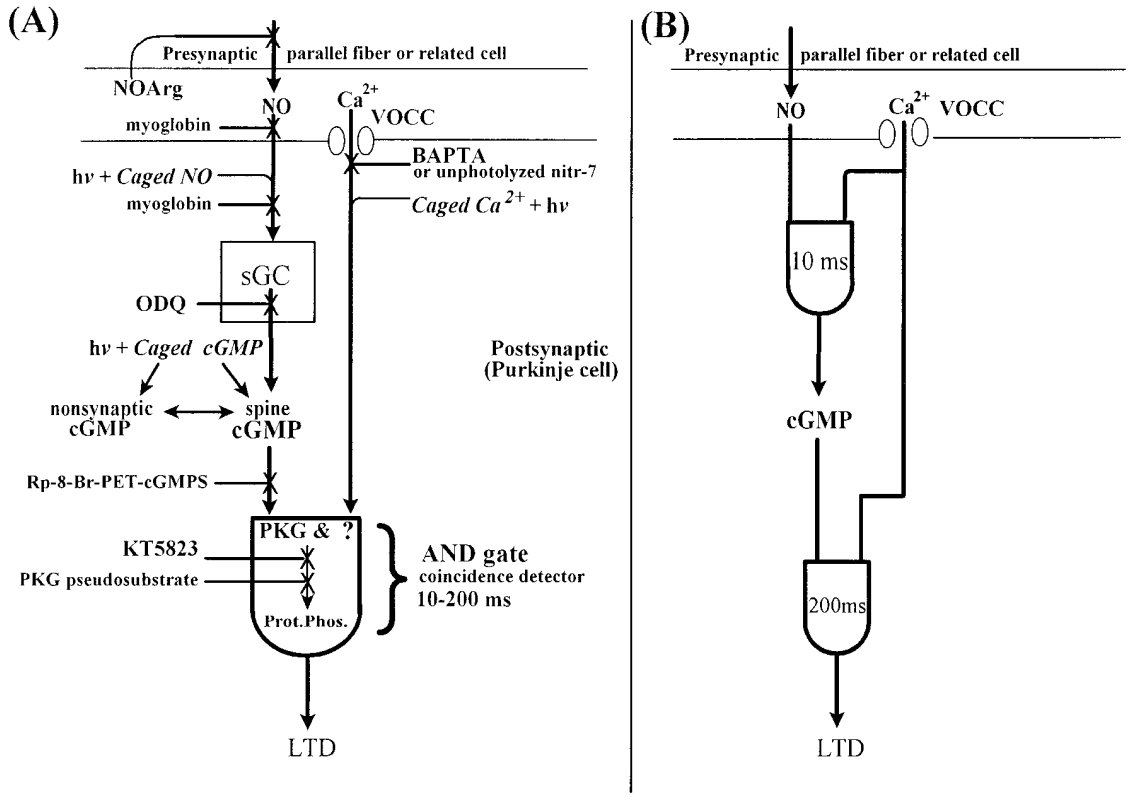

Figure 10. Alternative Hypotheses to Explain the Coincidence Requirements for Uncaged NO + Ca^{2+} versus Uncaged cGMP + Ca^{2+}

(A) A single molecular detector of coincidence between cGMP and Ca^{2+} (labeled as "AND" gate) could explain both the ~10 ms tolerance for NO + Ca^{2+} and the ~200 ms for cGMP + Ca^{2+} if the spatial profiles of cGMP differ as hypothesized in Figures 9B and 9C. Although PKG is drawn inside the "AND" gate because it is an essential target for cGMP, we do not know the actual effector for Ca^{2+} or how that molecule interacts with the cGMP pathway. The scheme also summarizes the various pharmacological inhibitors (pointing to x's interdicting their target pathways) and caged compounds (in italics) used in the work by Lev-Ram et al. (1995) and the present work to block and rescue LTD induction, respectively.

(B) An alternative hypothesis of two distinct coincidence detectors does not need to postulate differing spatial profiles of cGMP. The first coincidence detector allows a very short window of ~10 ms for NO and Ca^{2+} to coincide to give cGMP production. A second coincidence detector requires cGMP and Ca^{2+} but tolerates 200–300 ms gap. The inhibitors and caged compounds are the same as in (A) and are omitted for brevity.

obvious target for NO, sGC, is not Ca^{2+}-activated at physiologically plausible Ca^{2+} and Mg^{2+} concentrations (Olson et al., 1976; Mayer et al., 1992). The only clear case of a Ca^{2+}-dependent guanylate cyclase is in retinal photoreceptors, where the effect of raising $[Ca^{2+}]_i$ is inhibition rather than stimulation (Gorczyca et al., 1994).

The roles for NO, cGMP, and Ca^{2+} in the induction of Purkinje neuron LTD curiously parallel the initiation of LTP in hippocampal CA1 neurons. Such LTP requires coincidence of glutamate with depolarization to open NMDA receptors (Bliss and Collingridge, 1993), followed by coincidence of a retrograde signal such as NO with presynaptic activity (Arancio et al., 1996). Retrograde NO seems to act via presynaptic cGMP, which still needs presynaptic electrical activity to exert its effects (Zhuo et al., 1994; Arancio et al., 1995), but it remains to be seen whether such activity is simply a means for elevating $[Ca^{2+}]_i$ and if so, whether Ca^{2+} is synergizing with NO, cGMP, or both. Coincidence detection of Ca^{2+} and NO has been previously reported in the protein kinase A–dependent activation of gene expression in PC12 cells (Peunova and Enikolopov, 1993), though timing requirements were assessed only over timescales of minutes rather than milliseconds. Caged compounds permit analyses of timing windows with millisecond precision, which are powerful tools to dissect complex interactions of multistep signaling pathways.

Experimental Procedures

Thin (300 μm) sagittal slices were cut with a Microslicer DSK-3000W (D. S. K., Japan) from the cerebellar vermis of rats ages 18–21 days. Synaptic currents in Purkinje cells were recorded in the whole-cell patch-clamp configuration (Hamill et al., 1981; Edwards et al., 1989). The cells were directly visualized through a 10× water immersion objective on an upright microscope (Axioplan, Carl Zeiss Inc.). In some cases, the surfaces of Purkinje somata were gently cleaned using large-bore micropipettes. Tight-seal whole-cell recordings (seal resistance >10 gigaohms) were made with patch pipettes with 3–4 megohm resistance and an Axopatch 200A (Axon Instruments USA) amplifier (holding potential −70 mV). A 10 ms, 10 mV test depolarization preceded each PF stimulus to monitor the input resistance of the Purkinje cell throughout the experiment, which was discarded if the resistance changed by >20%. The currents produced by this test pulse are visible as the biphasic deflections at the beginning of some of the inset current traces. The intracellular solution contained (in mM): K-gluconate, 130; KCl, 10; K-HEPES, 10; $MgCl_2$, 1; Na–ATP, 4; Na–GTP, 1; sucrose, 16 [pH 7.2];

and osmolarity, 300 mOsm. Although these conditions are adequate for measuring excitatory postsynaptic currents (EPSCs), the voltage clamp cannot maintain space clamp conditions in the dendrites and axon when spikes are generated. The external Ringer's solution contained (in mM): NaCl, 125; KCl, 2.5; $CaCl_2$, 2; $MgCl_2$, 1; NaH_2PO_4, 1.25; $NaHCO_3$, 26; glucose, 25 [pH, 7.4]; and (-)-bicuculline methiodide, 10 μM (Research Biochemical International) to inhibit GABAergic synapses. Slices were continually perfused with Ringer's solution saturated with 95% O_2–5% CO_2 during recording. All experiments were performed at room temperature (near 22°C).

Intracellular calcium increases were achieved by 50 ms step depolarizations to a level in which regenerative calcium spikes were induced in the out-of-clamp dendritic region. For PF stimulation, a bipolar electrode was placed at the pial surface above and up to 200 μm on either side of the recorded PC. A 50 μs pulse was delivered to stimulate the PF using an isolation unit (ISO-Flex, A. M. P. I., Israel). The timing of depolarization, parallel fiber 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.

ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one) was purchased from Tocris Cookson Inc. (USA), LY85385 and KT5823 from Calbiochem (USA). Rp-8Br-PET-cGMPS was a gift from Biolog Life Sciences. The photosensitive Ca^{2+} chelator nitr-7 (Adams et al., 1988), the caged NO donor CNO-4 (Makings and Tsien, 1994), and the caged cGMPs DMNB-cGMP (guanosine 3′,5′-cyclic monophosphate, 4,5-dimethoxy-2-nitrobenzyl ester) (Nerbonne et al., 1984) and CM-cGMP (guanosine 3′,5′-cyclic monophosphate, 4,5-bis(carboxymethoxy)-2-nitrobenzyl ester) (T. J., and R. Y. T., unpublished data) were synthesized in this laboratory. NPE-cGMP (guanosine 3′,5′-cyclic monophosphate, P-1-[2-nitrophenyl] ethyl ester) was from Calbiochem. Caged compounds were introduced into the Purkinje neuron by inclusion in the patch pipette intracellular solution. To minimize electrical and mechanical disturbances, the photolytic illumination was provided by a 200 W DC mercury arc lamp with an electromechanical shutter (A. W. Vincent and Co.). The lamp was mounted separately from the main body of the microscope so that its collimated output entered the epifluorescence port through an air gap. Calibration experiments (Lev-Ram et al., 1995) indicated that the intensity of illumination at the plane of focus could release 2.8 μM NO per 200 ms irradiation period from droplets of 200 μM CNO-4 in buffer. The amount of NO released in cells would have been smaller due to tissue opacity to UV and any incompleteness of equilibration of the CNO-4 from patch pipette into the neuron. At the current UV intensity and CNO-4 concentration, the duration of efficacious uncaging flashes cannot be reduced much below 200 ms (Lev-Ram et al., 1995). The amounts of cGMP uncaged per 200 ms irradiation period from 75 μM DMNB-cGMP or 400 μM CM-cGMP are estimated to be 0.32 and 1.7 μM, respectively, based on their extinction coefficients and quantum yields (Wootton and Trentham, 1989). Likewise, each 500 ms illumination of the nitr-7 should convert about 22% of the Ca^{2+}-bound nitr-7 (K_d for Ca^{2+} = 54 nM) to the low affinity (K_d = 3 μM) nitrosobenzhydrol. The magnitude of each jump in free Ca^{2+} cannot be calculated precisely because we do not know the starting degree of Ca^{2+} loading, which depends on the previous Ca^{2+} channel verses pump activity of the cell, and because diffusion of Ca^{2+}-free nitr-7 from the pipette will be occurring meanwhile. Nevertheless, 30 flashes at 1 Hz are expected to photolyze practically all of the nitr-7 in the cell and cumulatively raise the $[Ca^{2+}]_i$ to μM levels. Additional photolysis periods can repeat the elevation if a sufficient period is allowed for diffusional exchange of unphotolyzed nitr-7 from the pipet.

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