Tests of the roles of two diffusible substances in long-term potentiation: Evidence for nitric oxide as a possible early retrograde messenger

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ABSTRACT Although long-term potentiation (LTP) in the CA1 region of the hippocampus is initiated postsynaptically by the influx of Ca\(^{2+}\) through N-methyl-D-aspartate receptor channels, the maintenance of LTP seems to be at least in part presynaptic. This suggests that the postsynaptic cell releases a retrograde messenger to activate the presynaptic terminals. It is likely that this messenger is membrane-permeant and reaches the presynaptic neuron by diffusion. We therefore have investigated two major membrane-permeant candidates: retrograde messengers, arachidonic acid and nitric oxide (NO). Consistent with arachidonic acid or a lipoxygenase metabolite being a retrograde messenger, the phospholipase A\(_2\) and lipoxygenase inhibitor nordihydroguaiaretic acid blocked LTP in the guinea pig CA1 region in vitro. However, arachidonic acid (up to 100 \(\mu\)M) did not reliably produce activity-dependent LTP, and activity-dependent potentiation by arachidonic acid was blocked by DL-aminophosphonovaleric acid. Since nordihydroguaiaretic acid also interferes with signal transduction involving NO, we next examined whether inhibitors of NO synthase block LTP. \(N^2\)-Nitro-L-arginine blocked LTP when given in the bath, and this inhibition was partially overcome by high concentrations of L-arginine, suggesting that the inhibitor is specific to NO synthase. \(N^2\)-Nitro-L-arginine and \(N^2\)-methyl-L-arginine (but not \(N^2\)-methyl-D-arginine) also blocked LTP when injected intracelullarily, indicating that NO synthase is located in the postsynaptic cell. The NO, in turn, seems to be released into the extracellular space, since bathing the slice with hemoglobin, a protein that binds NO and is not taken up by cells, also blocked LTP. Moreover, NO enhances spontaneous presynaptic release of transmitter from hippocampal neurons in dissociated cell culture. These data favor the idea that NO might be a retrograde messenger in LTP.

Synapses in the CA1 region of the hippocampus display a Hebbian form of long-term potentiation (LTP). The induction of LTP involves the activation of the N-methyl-D-aspartate (NMDA)-type glutamate receptors during conjunctive pre- and postsynaptic activity and the consequent increase in Ca\(^{2+}\) influx into the postsynaptic cells (1–3). Whereas a number of Ca\(^{2+}\)-activated biochemical processes in the postsynaptic neurons are thought to underlie LTP (4–6), the maintenance of LTP is thought to involve enhanced transmitter release from the presynaptic terminals (7–10). The localization of different phases of LTP to first post- and then presynaptic neurons suggests that the induction of LTP involves the production, and release from the dendrites of the postsynaptic cell, of a retrograde messenger that acts on the presynaptic terminals. Since the postsynaptic dendritic spines do not have the conventional machinery for the release of transmitter, we have assumed that the retrograde messenger is membrane-permeant and reaches the presynaptic terminals by free diffusion (11). We therefore have begun to examine two molecules that are freely diffusible and that might serve as retrograde messengers: arachidonic acid (11, 12) and nitric oxide (NO) (13, 14).

METHODS

Hippocampal Slices. LTP in the CA1 region of slices of guinea pig hippocampus was studied by methods described elsewhere (15). Briefly, we stimulated the Schaffer collateral/commissural fibers and recorded excitatory postsynaptic potentials (EPSPs) with either an extracellular electrode in the stratum radiatum or an intracellular electrode. In experiments with an intracellular electrode, picrotoxin was used to suppress inhibitory postsynaptic potentials, the concentrations of CaCl\(_2\) and MgSO\(_4\) were both increased to 4.0 mM, and the CA3 region of the hippocampus was removed to prevent epileptiform activity. To minimize spurious results from slices that were damaged, the effects of inhibitors delivered via the intracellular microelectrode were examined only in slices where LTP was first demonstrated in other cells of the same slice without the inhibitor in the electrode solution. In all experiments with inhibitors in the electrode solution, LTP was induced after waiting at least 30 min after impaling the cell to allow diffusion of the inhibitor into the cell.

Stock solutions of arachidonic acid (50 mM in dimethyl sulfoxide) were stored under argon gas at −70°C and used in less than 8 hr. Just prior to application, stock solutions of arachidonic acid were thawed and dissolved in artificial cerebrospinal fluid containing 100 \(\mu\)M ascorbic acid by sonication. Hemoglobin was prepared from methemoglobin as described by Martin et al. (16).

Hippocampal Dissociated Cell Culture. Hippocampi from 1- to 2-day newborn rats (Sprague–Dawley strain) were dissociated through enzymatic treatment (0.25% trypsin) and subsequent trituration. The cells were plated on glass coverslips previously coated with poly(t-lysine) and laminin. Hippocampal cells were grown in medium containing 84% Eagle’s minimum essential medium (MEM), supplemented with 10% heat-inactivated fetal calf serum, 45 mM glucose, 1% MEM vitamin solution, and 2 mM glutamine. After 24 hr this medium was replaced by a medium containing 1% heat-inactivated fetal calf serum and 0.5 mM kynurenic acid (17).

In preparation for recording, a coverslip with hippocampal neurons grown for 6–13 days in culture was placed in a Lucite

Abbreviations: APV, DL-aminophosphonovaleric acid; EPSP, excitatory postsynaptic potential; LTP, long-term potentiation; mEPSC, miniature excitatory postsynaptic current; NDGA, nordihydroguaiaretic acid; NMDA, N-methyl-D-aspartate; NOArg, \(N^2\)-nitro-L-arginine.

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chamber on the stage of an inverted microscope. Bath solution (composition in mM, 119 NaCl, 5 KCl, 20 Hepes, 2 CaCl₂, 2 MgCl₂, 30 glucose, 10⁻³ tetrodotoxin, 10⁻³ glycine, pH 7.3; 310 milliosmolar, 24°C) was continuously oxygenated and perfused through the chamber at 1 ml/min. In some experiments 50 μM DL-aminophosphonovaleric acid (APV) was added to the bath. Recordings were made from neurons that were, in most cases, pyramidal shaped, using the whole-cell patch-clamp technique under voltage clamp (holding potential, −60 mV). Recording pipettes (10 MΩ) were filled with (in mM) 110 cesium gluconate, 10 KCl, 5 Hepes, 0.6 EGTA, and 5 MgCl₂ (pH 7.1, 300 milliosmolar adjusted with sucrose).

NO was prepared by bubbling the gas until saturation in helium-saturated distilled water (18). Immediately before the application NO was diluted in bath solution without MgCl₂ to reach a final concentration of 5–10 nM. The chamber was perfused with NO solution for approximately 30 sec. In control experiments the same solution containing helium but free of NO was applied.

**RESULTS**

Arachidonic Acid as a Candidate Retrograde Messenger in LTP. Consistent with the idea that arachidonic acid or one of its lipoxygenase metabolites serves as a retrograde messenger in LTP, we found, as has been reported by others (19, 20), that bath application beginning 30 min pretetanus of nordihydroguaiaretic acid (NDGA; 100 μM in 0.1% dimethyl sulfoxide), an inhibitor of the lipoxygenase and phospholipase A₂, blocked LTP, while indomethacin (100 μM in 0.1% dimethyl sulfoxide), a cyclooxygenase inhibitor, did not. However, a 30-min bath application of 100 μM arachidonic acid had no consistent effect on the magnitude of the extracellularly recorded EPSPs evoked by Schaffer collateral/commisural fiber stimulation (Fig. 1A; see also ref. 21).

Since Williams et al. (12) reported that the effects of arachidonic acid in the dentate gyrus of the hippocampus are activity dependent, we examined whether weak tetanic stimulation (50 Hz, 0.5 sec) that usually failed to produce long-lasting potentiation when given alone (Fig. 1B and ref. 22) produced a persistent enhancement of synaptic transmission when delivered in the presence of arachidonic acid. Pairing a 30-min application of 100 μM arachidonic acid with a 50-Hz tetanus of 0.5-sec duration led to an enhancement in CA1 that was significantly larger than the effect of tetanus alone (t = 5.67, P < 0.01, 60 min after tetanus) (Fig. 1C). Because the release of the endogenous retrograde messenger during the induction of LTP must occur at a step subsequent to the activation of the NMDA receptor, the potentiating effect of an exogenously applied candidate retrograde messenger should be independent of the activation of NMDA receptors. However, we found that 50 μM APV prevented the potentiation produced by arachidonic acid paired with the tetanus (Fig. 1D). Thus, although inhibitors of arachidonic acid metabolism can block LTP, the results from experiments in which we applied exogenous arachidonic acid are not consistent with a role for arachidonic acid as an immediate retrograde messenger for LTP in CA1.

**Inhibitors of NO Synthase Block LTP.** Since NDGA also interferes with the NO signal transduction pathway (23), we next examined whether LTP is blocked by inhibitors of NO synthase, the enzyme that synthesizes NO and citrulline from the amino acid arginine. Indeed, we found that LTP was blocked (Fig. 2A) by concentrations of NG⁰-nitro-L-arginine (NOArg) that block NMDA-stimulated increases in cGMP in the hippocampus (24) (t = 8.10, P < 0.01, compared with control). This inhibition appears to be specific, since it was partially reduced by 1 mM L-arginine, the substrate for NO synthase (Fig. 2B).

If NO is synthesized postsynaptically, it should be possible to block LTP when the inhibitors of NO synthase are injected directly into the CA1 pyramidal cells. To investigate this question, we impaled the CA1 neurons with an intracellular microelectrode and induced LTP by pairing EPSPs with postsynaptic membrane depolarization. Pairing 15–20 EPSPs delivered at low frequency (1.0 Hz) with depolarization of the postsynaptic cell to near 0 MV reliably (about 70% of the time) elicited a robust potentiation of synaptic transmission (Fig. 3A). LTP so induced was significantly reduced, however, when cells were impaled with electrodes containing 10 mM NOArg (t = 6.70, P < 0.01) (Fig. 3B) or N⁰-methyl-L-arginine (t = 13.29, P < 0.01) (Fig. 3C). The inactive analog N⁰-methyl-d-arginine, which does not block brain NO synthase (25), did not block LTP (Fig. 3D). These experiments suggest that the enzyme is located in the postsynaptic cell.

**Fig. 1.** Activity-dependent effects of arachidonic acid (ARAC) on synaptic transmission are blocked by APV. (A) A 30-min bath application of 100 μM arachidonic acid (0.2% dimethyl sulfoxide/100 μM ascorbate) had no consistent effect on EPSPs elicited by 0.02-Hz Schaffer collateral/commisural fiber stimulation (n = 5). (B) A 50-Hz, 0.5-sec tetanus (delivered at time = 0) on average elicits only a small potentiation (n = 6). (C) The combination of this tetanus with a 30-min bath application of 100 μM arachidonic acid (0.2% dimethyl sulfoxide/100 μM ascorbate) starting 20 min prior to tetanic stimulation produces a significantly larger potentiation than tetanus alone (n = 6). (D) APV blocks the potentiation produced by pairing arachidonic acid and 50-Hz, 0.5-sec tetanus (n = 5). Slices were bathed in 50 μM APV throughout the experiment. Values plotted are mean ± SEM.
NO would be expected to produce an increase in presynaptic transmitter release. To test this possibility, we have performed experiments on hippocampal neurons in dissociated cell culture, where it is possible to observe spontaneous mEPSCs. A change in the frequency of mEPSCs is indicative of a presynaptic effect on the release process, whereas a change in the amplitude of mEPSCs is usually indicative of a postsynaptic effect. Brief application of glutamate (in the presence of low magnesium and tetrodotoxin) produces a long-lasting increase in the frequency of spontaneous mEPSCs in dissociated cell culture that resembles LTP in that it is blocked by an NMDA receptor blocker or by hyperpolarizing the postsynaptic cell during application of the glutamate (26). In preliminary experiments we confirmed this finding.

We next tested whether application of exogenous NO produces a similar increase in the frequency of spontaneous mEPSCs in hippocampal neurons in dissociated cell culture, using the whole-cell patch-clamp technique under voltage clamp. Application of NO at concentrations of 40 nM or higher led to cessation of spontaneous mEPSCs followed by cell death. However, brief application of 5–10 nM NO produced a rapid and sustained increase in the frequency of spontaneous mEPSCs (Fig. 4). One minute after NO application, mEPSC frequency was significantly elevated compared to pre-application baseline (t = 1.95, P < 0.05, one-tail) and vehicle control (t = 2.12, P < 0.5, two-tail). mEPSC frequency was still significantly elevated over baseline 30 min after NO application (t = 3.23, P < 0.05, one-tail), and in two experiments that lasted over 60 min mEPSC frequency was still elevated at that time (geometric mean = 223% of Pre). Controls that received solution showed no increase in mEPSC frequency.

If NO were to serve as a retrograde messenger, its actions should be independent of NMDA receptor activation, since application of exogenous NO would bypass that step. Application of NO produced no detectable change in the holding current in the postsynaptic cell, suggesting that it does not activate NMDA receptors. Moreover, brief application of NO in the presence of 50 μM APV still produced an increase in mEPSC frequency lasting at least 60 min (234% of Pre, n = 1), whereas application of vehicle had no effect (89% of Pre, n = 1).

The persistent increase in mEPSC frequency far outlasts the brief application of NO, supporting the idea that NO needs to act only transiently as a retrograde messenger and that the maintenance of LTP is, to an important degree, presynaptic. In two of three experiments lasting more than 30 min, we also observed a delayed increase in the amplitude of the mEPSCs, suggesting that in the later stages of LTP there may also be a postsynaptic increase in receptor sensitivity or a change in presynaptic release characteristics.

**DISCUSSION**

To develop criteria for identifying a retrograde messenger, we have been guided by those that have proven useful in the identification of molecules released in normal, anterograde, synaptic transmission, as well as the known properties of LTP in the CA1 region of the hippocampus. We have thus considered the following nine requirements for a retrograde messenger: (i) The messenger should be synthesized by the postsynaptic CA1 pyramidal cells. (ii) It should be released in response to the activation of NMDA receptors during the induction of LTP. (iii) Inhibiting the synthesis of the retrograde messenger should block LTP. (iv) There should be a pathway for removing or degrading the active messenger. (v) Exogenous application of the candidate retrograde messenger should mimic LTP. (vi) The actions of an exogenously applied candidate messenger should be independent of the NMDA receptor, since during the induction of LTP the release of the endoge-

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**Fig. 2.** Inhibitors of NO production and release block LTP. (A) NOArg applied (see bar at top of B) at 50 μM for 40 min beginning 30 min prior to tetanic stimulation (two 100-Hz trains, 1-sec duration, separated by 30 sec) blocks LTP (n = 6). Application of 50 μM NOArg beginning 30 min after tetanus had no effect on established LTP (n = 3, data not shown). Control LTP (Δ, n = 6) is shown for comparison. (B) Arginine (1 mM) reduces the NOArg inhibition of LTP. Experiments were performed as in A except 1 mM l-arginine was present throughout (n = 7). (C) Bath application of hemoglobin, but not methemoglobin, reduces LTP. Artificial cerebrospinal fluid containing 20 μM hemoglobin (Δ, n = 9) was perfused for at least 1 hr prior to attempting to elicit LTP by tetanic stimulation. Methemoglobin (20 μM) applied for similar time periods did not block LTP (Δ, n = 6).

For NO to act as a retrograde messenger, it must diffuse from the postsynaptic cell into the extracellular space of the synaptic cleft to act on its presynaptic target. To examine whether NO is present extracellularly, we bathed slices with hemoglobin, a protein that binds NO and does not penetrate cell membranes, and found that 20 μM hemoglobin applied for 1–5 hr pretetanus blocked LTP (t = 10.01, P < 0.01) (Fig. 2C). Bath application of 20 μM methemoglobin, which has a much lower affinity for NO (14), had no effect on LTP.

**NO Increases the Frequency of Spontaneous Miniature Excitatory Postsynaptic Currents (mEPSCs).** If NO acts as a retrograde messenger in LTP, then application of exogenous
uous retrograde messenger presumably occurs after NMDA receptor activation. (vi) The actions of the retrograde messenger should be rapid, since the observed increased release of neurotransmitter during the expression of LTP occurs immediately after its induction (8, 9). (vii) Other types of synaptic facilitation thought to occur presynaptically (such as paired-pulse facilitation) should not be occluded by the potentiation produced by the retrograde messenger (27). (viii) The retrograde messenger should be synapse specific, since during LTP potentiation occurs only at synapses of the stimulated presynaptic fibers and not other fibers. Synapse specificity may be achieved either by (i) spatially restricted diffusion of a locally released messenger, whereby the retrograde messenger should have actions independent of activity in the presynaptic terminals, or by (ii) activity-dependent actions of the retrograde messenger, whereby activity in the presynaptic terminals renders them receptive to the influence of the retrograde messenger (see ref. 28).

Arachidonic acid satisfies many, but not all, of the above criteria at the perforant pathway to granule cell synapses in the dentate gyrus (see ref. 7 for review). However, we find that it does not fulfill as many of these criteria as the Schaffer collateral synapse onto CA1 pyramidal cells. The evidence for NO as a retrograde messenger is more compelling, but this evidence is also not complete. Consistent with a role of NO in LTP, inhibitors of NO synthase block the induction of LTP.

FIG. 3. LTP produced by pairing postsynaptic depolarization with low-frequency stimulation of the presynaptic fibers (bar) is inhibited by 10 mM N0Arg and N0 methyl-L-arginine but not N0 methyl-L-arginine in the electrode solution. See text for details. The number of cells in each average is 21, 10, 11, and 10 for A, B, C, and D, respectively. The traces at the top of A and C show responses elicited just prior to and 60 min after pairing EPSPs with postsynaptic depolarization. Calibration bars (in A) are 12 msec and 5.0 mV. EPSPs evoked 1 hr after depolarization alone with N0 methyl-L-arginine-containing electrodes were 102.9 ± 14.4% of control (n = 5), suggesting that the intracellular delivery of N0 methyl-L-arginine does not inhibit normal, unpotentiated synaptic transmission.

FIG. 4. NO produces a rapid and sustained increase in the frequency of spontaneous mEPSCs. (A) mEPSCs recorded from a hippocampal neuron in dissociated cell culture before (Pre), 1 min, and 30 min after brief application of 5 nM NO in a representative experiment. (B) Average change in mEPSC frequency in all experiments. NO (●) or vehicle (control, □) was applied at time zero (arrow). mEPSC frequency has been normalized to the average value during the 4 min before the application (Pre) in each experiment (geometric mean = 49 min−1 for NO and 41 min−1 for control, not significantly different). Error bars indicate the geometric mean and SEM, the numbers in parentheses indicate the n at each time point, and asterisks indicate a significant difference from the Pre level.
slices (29). These observations suggest that NO is produced postsynaptically and diffuses to act on the presynaptic cells during the induction of LTP, consistent with its possible role as a retrograde messenger. NO in turn could act on one of two targets in the presynaptic cell, soluble guanylate cyclase and ADP ribosyltransferase (Fig. 5). The ribosyltransferase is a particularly intriguing target, since it could produce long-lasting covalent modification of channel proteins or of other molecular steps in the release pathway.

On the other hand, little or no brain-specific NO synthase has been found as yet in CA1 pyramidal cells (31, 32), although other isoforms of the enzyme could be present and generate NO. It is also not yet known whether exogenous NO [and not a by-product remaining after the release of NO from nitroprusside (29)] can potentiate evoked postsynaptic potentials in slice or in culture. Thus, the identification of NO as a retrograde messenger must be considered tentative until there are demonstrations that NO can be synthesized in these cells and that exogenously supplied NO can simulate completely the natural induction of LTP.

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