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Nitric Oxide Acts as a Postsynaptic Signaling Molecule in Calcium/Calmodulin-Induced Synaptic Potentiation in Hippocampal CA1 Pyramidal Neurons

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Postsynaptic injection of Ca^{2+}/calmodulin (Ca^{2+}/CaM) into hippocampal CA1 pyramidal neurons induces synaptic potentiation, which can occlude tetanus-induced potentiation (Wang and Kelly, 1995). Because Ca^{2+}/CaM activates the major forms of nitric oxide synthase (NOS) to produce nitric oxide (NO), NO may play a role during Ca^{2+}/CaM-induced potentiation. Here we show that extracellular application of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) or postsynaptic co-injection of L-NAME with Ca^{2+}/CaM blocked Ca^{2+}/CaM-induced synaptic potentiation. Thus, NO is necessary for Ca^{2+}/CaM-induced synaptic potentiation. In contrast, extracellular perfusion of membrane-impermeable NO scavengers *N*-methyl-D-glucamine dithiocarbamate/ferrous sulfate mixture (MGD-Fe) or 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) did not attenuate Ca^{2+}/CaM-induced synaptic potentiation, even though MGD-Fe or carboxy-PTIO blocked tetanus-induced synaptic potentiation. This result indicates that NO is not a retrograde messenger in Ca^{2+}/CaM-induced synaptic potentiation. However, postsynaptic co-injection of carboxy-PTIO with Ca^{2+}/CaM blocked Ca^{2+}/CaM-induced potentiation. Postsynaptic injection of carboxy-PTIO alone blocked tetanus-induced synaptic potentiation without affecting basal synaptic transmission. Our results suggest that NO works as a postsynaptic (intracellular) messenger during Ca^{2+}/CaM-induced synaptic potentiation.

Key words: nitric oxide; calmodulin; hippocampus; synaptic plasticity; synaptic potentiation; nitric oxide scavengers; nitric oxide synthase inhibitors

The role of nitric oxide (NO) as a retrograde messenger in hippocampal long-term potentiation (LTP) has been suggested by many studies (O'Dell et al., 1991; Schuman and Madison, 1991, 1994a,b; Haley et al., 1992; Bredt and Snyder, 1994; Garthwaite and Boulton, 1995; Arancio et al., 1996). Extracellular application of NO donors facilitates LTP induction (Malen and Chapman, 1997), whereas extracellular administration of nitric oxide synthase (NOS) inhibitors or NO scavengers or postsynaptic injection of NOS inhibitors attenuates and/or blocks LTP induced by tetanus or pairing protocols (Haley et al., 1992; Williams et al., 1993a; Schuman and Madison, 1994a; Arancio et al., 1996) (but see Chetkovitch et al., 1993; Cummings et al., 1994). Two major forms of constitutive NOS in brain are neuronal NOS and endothelial NOS (nNOS and eNOS, respectively) (Bredt and Snyder, 1994), and both are present in the hippocampus (Dinerman et al., 1994; Doyle and Slater, 1997; Eliasson et al., 1997). Mutant mice lacking nNOS and eNOS display significantly attenuated hippocampal LTP (Son et al., 1996).

Previous results have shown that postsynaptic injection of Ca^{2+}/calmodulin (Ca^{2+}/CaM) induces synaptic potentiation in hippocampal CA1 neurons (Wang and Kelly, 1995). Ca^{2+}/CaM-induced synaptic potentiation is similar to tetanus-induced LTP because it is blocked by co-injecting a calmodulin binding peptide or pseudosubstrate inhibitors of Ca^{2+}/CaM-dependent kinase II (CaMKII) or protein kinase C (PKC) (Wang and Kelly, 1995). Tetanus-induced LTP and Ca^{2+}/CaM-induced synaptic potentiation reciprocally occlude each other, so their underlying mechanisms may be similar (Wang and Kelly, 1995). Because mechanisms responsible for tetanus-induced LTP expression are believed to be in part presynaptic (Malinow and Tsien, 1990a; Bolshakov and Siegelbaum, 1995), it is important to determine whether Ca^{2+}/CaM-induced synaptic potentiation involves a presynaptic mechanisms(s), particularly because the latter is induced by an apparently restricted postsynaptic manipulation. For example, postsynaptic injection of Ca^{2+}/CaM could activate n/eNOS and elevate NO in neurons (Bredt and Snyder, 1990; Bredt et al., 1992; Brenman et al., 1996), and NO could act as a retrograde messenger during Ca^{2+}/CaM-induced synaptic potentiation. Alternatively, NO could contribute to Ca^{2+}/CaM-induced synaptic potentiation by acting locally in postsynaptic neurons to directly nitrosylate and/or oxidize proteins (Lei et al., 1992; Lipton et al., 1993, 1996; Li et al., 1999) or indirectly via the activation of guanylyl cyclase/cyclic GMP-dependent protein kinase and/or ADP-ribosyl transferase pathways (Boulton et al., 1995; Schuman et al., 1994; Zhou et al., 1994a,b; Kleppisch et al., 1999).

We examined the role of NOS and NO in synaptic potentiation induced by postsynaptic injection of Ca^{2+}/CaM. We also investigated whether NO acted as a retrograde messenger and/or a postsynaptic signaling molecule in Ca^{2+}/CaM-induced synaptic potentiation. Tetanus-induced synaptic potentiation was monitored as an index for the effectiveness of an NOS inhibitor or NO

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scavengers. Here we show that postsynaptic NO is involved in Ca^{2+}/CaM-induced potentiation but not as a retrograde messenger. On the other hand, we observed that tetanus-induced potentiation appears to require NO-dependent retrograde signaling, consistent with previous observations (O'Dell et al., 1991; Schuman and Madison, 1991, 1994a; Haley et al., 1992; Arancio et al., 1996; Malen and Chapman, 1997).

MATERIALS AND METHODS

Male and female Sprague Dawley rats (35–55 days old; from Harlan Sprague Dawley, Indianapolis, IN; and Charles River, Wilmington, MA) were used in this study. Animals were group-housed and maintained in a temperature-controlled environment with a 12 hr light/dark cycle. Transverse hippocampal slices (400 μm) were prepared using a McIlwain tissue chopper with ice-cold modified artificial CSF (ACSF). Slices were incubated at 25 °C in ACSF over 1 hr and transferred to a submersion chamber (31.8 ± 0.5 °C; 2.5–3 ml/min perfusion rate) for electrophysiological recordings. ACSF contained (in mM): 124 NaCl, 3 KCl, 1.3 NaH_2PO_4, 26 $NaHCO_3$, 2.0 $MgCl_2$, 2.4 $CaCl_2$, 10 glucose, and 10 HEPES, pH 7.3. A modified ACSF used for slice preparation contained 4.0 mM $MgCl_2$ and 1.2 mM $CaCl_2$. All media were bubbled with a 95%O_2-5%CO_2 mixture. Experiments were conducted in "standard" ACSF containing bicuculline (5 μM), and the concentration of $MgCl_2$ was 2.4 mM. Isolated CA1 slices were made by cutting presynaptic axons in stratum radiatum but not in oriens/alveus at the CA1–CA3 border. Under these conditions, seizure activity was never observed during basal synaptic transmission. Complex waveforms only occurred after tetanic stimulation in some experiments. In designated experiments, the perfusion medium also contained 100 μM N^G-nitro-L-arginine methyl ester (L-NAME), a mixture of 150 μM *N*-methyl-D-glucamine dithiocarbamate and 75 μM $FeSO_4 \cdot 7\ H_2O$ (150/75 μM MGD-Fe), or 30 μM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO). Constant flow rates were maintained throughout experiments especially during media exchanges.

Glass microelectrodes (60–85 MΩ) filled with 2 M potassium acetate (KAc), carboxy-PTIO in 2 M KAc, Ca^{2+}/CaM/KAc, or Ca^{2+}/CaM/KAc plus various agents [i.e., L-NAME, N^G-nitro-D-arginine methyl ester (D-NAME), or carboxy-PTIO] were used for intracellular recordings in CA1 pyramidal neurons in bridge mode. Ca^{2+}/CaM/KAc was prepared from $CaCl_2$, CaM, and KAc stocks to obtain final concentrations of 80 and 20 μM and 2 M, respectively. Input resistance was estimated by injecting negative current (0.12 nA) for 50 msec before each evoked stimulus and monitored throughout recordings. Results were only collected from neurons in which stable recordings were obtained within the initial 2–5 min after impalement with resting membrane potentials between −65 and −73 mV, and in which input resistance changed <20% throughout the entire experiment. Extracellular field EPSPs were recorded using glass pipettes (containing 0.9% NaCl) placed below the intracellular recording site (halfway between stratum pyramidale and the hippocampal fissure). One bipolar tungsten stimulating electrode (~12 MΩ) was placed in CA1 stratum radiatum for orthodromic stimulation of Schaffer collateral/commissural fibers. Test stimuli were given at 0.05 Hz, and stimulus intensity was adjusted to evoke about one-half to three-fifths of maximal synaptic responses. Tetanic stimulation was delivered at 100 Hz (five trains of 25 pulses at 5 sec intervals) and the same stimulus intensity used to evoke baseline responses. Intracellular and extracellular recordings, data acquisition, and analysis were performed using an AxoClamp 2B amplifier with Axoscope and Clampfit softwares (Axon Instruments). Initial baseline values were averaged from EPSP slopes obtained during the first 1–2 min after intracellular recordings stabilized and defined as 100%. Values of tetanus- or Ca^{2+}/CaM-induced synaptic potentiation, or different drug treatments, were obtained from data points averaged over a 2 min period at the time indicated (e.g., 45 min after beginning intracellular injection). Student's *t* tests were used for comparisons within the same experimental groups at different times (paired *t* test) and for comparisons between different groups at comparable experimental times (nonpaired *t* test). Values were expressed as mean ± SEM; significant differences were determined at the $p < 0.05$ level.

Bicuculline methbromide was obtained from Research Biochemicals (Natick, MA), CaM from Calbiochem (La Jolla, CA), L-NAME and D-NAME from Sigma (St. Louis, MO), carboxy-PTIO from Cayman (Ann Arbor, MI); MGD and $FeSO_4.7H_2O$ were gifts from Dr. Yashige Kotake (Oklahoma Medical Research Foundation, Oklahoma City, OK); chemicals for ACSF were from Fisher Scientific (Fair Lawn, NJ).

RESULTS

Postsynaptic injection of Ca^{2+}/CaM induces synaptic potentiation

Intracellular recordings using sharp microelectrodes with simultaneous extracellular field recordings were used to monitor synaptic transmission of hippocampal CA1 neurons. Postsynaptic injections of Ca^{2+}/CaM (80 and 20 μM, respectively) into CA1 pyramidal neurons by passive diffusion from microelectrodes induced a gradual increase in the initial slopes of EPSPs (Fig. 1*A*). Initial baseline values were averaged from six consecutive EPSPs during the first 1–2 min after intracellular recordings stabilized and defined as 100%. Figure 1*A* shows that postsynaptic injection of Ca^{2+}/CaM into CA1 pyramidal neurons for 45 min induced significant synaptic potentiation of EPSP slopes (184 ± 19%; $n = 5$; 45 min after beginning intracellular injections) compared with initial baseline values ($p < 0.05$). These results are consistent with previous studies showing that postsynaptic injection of Ca^{2+}/CaM enhanced excitatory synaptic transmission (Wang and Kelly, 1995). Simultaneous extracellular field recordings showed a stable baseline during the pretetanus period (105 ± 6%; $n = 5$; 45 min after beginning intracellular injections; Fig. 1*B*), whereas intracellular injections displayed Ca^{2+}/CaM induced potentiation. The magnitude of Ca^{2+}/CaM-induced potentiation decreased after 100 Hz tetanic stimulation (25 pulses, five trains at 5 sec intervals). This depotentiation was previously observed; however, the underlying mechanism is not known (Wang and Kelly, 1995). In contrast, field recordings showed significant potentiation induced by tetanus (155 ± 15% at 30 min after tetanus; $n = 5$, Fig. 1*B*; 148 ± 21% at 60 min after tetanus; results not shown). A control group was included, in which microelectrodes were filled only with 2 M KAc. After recording stable baselines for 20 min, tetanus was delivered, which induced significant synaptic potentiation in both intracellular (198 ± 16% at 30 min after tetanus; $n = 4$; Fig. 1*C*; $p < 0.05$) and field EPSP slopes (167 ± 11% at 30 min after tetanus; $n = 4$; Fig. 1*D*; $p < 0.05$) compared with pretetanus baseline values.

Extracellular NOS/NO modulators block tetanus-induced synaptic potentiation

Because our ultimate goal was to determine whether NO signaling pathways contribute to Ca^{2+}/CaM-induced synaptic potentiation, we needed an independent measurement of the efficacy of NO modulators in our experiments. Previous studies indicated that extracellular application of NOS inhibitors or NO scavengers blocked tetanus-induced potentiation in hippocampal CA1 area (Schuman and Madison, 1991, 1994a; Haley et al., 1992). To investigate whether NOS/NO modulators would affect basal synaptic transmission or block tetanus-induced potentiation, the following experiments were conducted using extracellular field recordings. Stable baselines (field EPSPs) were recorded for at least 20–30 min, and then extracellular perfusions of an NOS inhibitor or NO scavenger were initiated. We used recently developed NO scavengers MGD and carboxy-PTIO instead of hemoglobin. Extracellular perfusion of hemoglobin can depolarize CA1 neurons and suppress EPSPs and IPSPs in the presence of the NOS inhibitor *N*-ω-nitro-L-arginine, suggesting that hemoglobin has other effects that are independent of its NO scavenging activity (Yip et al., 1996). MGD mixed with reduced iron (Fe^{2+}) forms a stable and water-soluble complex (MGD-Fe; Komarov et al.,

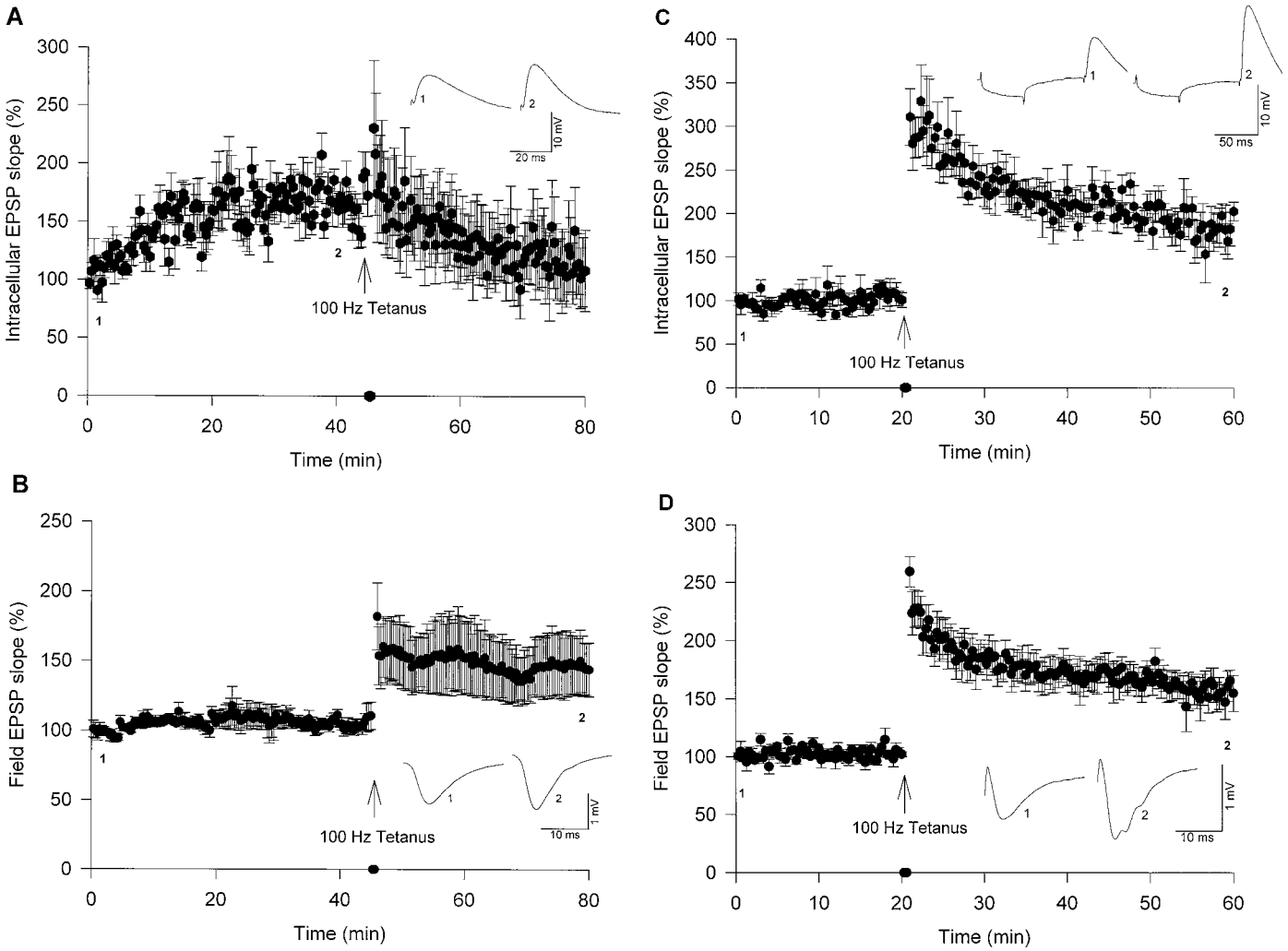

Figure 1. Postsynaptic injection of Ca^{2+}/CaM induces synaptic potentiation. *A*, *B*, Ca^{2+}/CaM was injected for 45 min, and then a 100 Hz tetanus was delivered. *A*, Synaptic potentiation induced by postsynaptic Ca^{2+}/CaM injections ($n = 5$). *B*, Simultaneous field recordings displayed stable baseline EPSPs followed by tetanus-induced synaptic potentiation ($n = 5$). *C*, *D*, Tetanic stimulation under control conditions induced synaptic potentiation. *C*, Intracellular recordings, microelectrodes filled with 2 M KAc ($n = 4$). *D*, Simultaneous field recordings of control group ($n = 4$).

1993), which is cell membrane-impermeable (Y. Kotake, personal communication) and has been used for *in vivo* spin trapping of NO in mice (Lai and Komarov, 1994; Komarov and Lai, 1995; Kotake et al., 1995). MGD-Fe has also been used to scavenge NO produced by cells in culture (Kotake, 1996; Kotake et al., 1996) or *in vitro* cardiovascular preparations (Pieper and Lai, 1996). Perfusion of freshly mixed MGD and $FeSO_4$ (i.e., MGD-Fe) at different concentrations was tested (data not shown). MGD-Fe mixtures of 100/25, 100/37.5, and 100/50 μM did not block tetanus-induced potentiation; however, MGD-Fe at 150/75 μM successfully blocked tetanus-induced potentiation without affecting basal synaptic transmission (see below).

We also tested carboxy-PTIO, which is water-soluble and scavenges NO without affecting NOS activity (Az-ma et al., 1994; Maeda et al., 1994; Yoshida et al., 1994; Amano and Noda, 1995; Hogg et al., 1995a,b). Preliminary results in cultured cells indicate that carboxy-PTIO is cell membrane-impermeable (T. Akaike, personal communication). Extracellular perfusions of carboxy-PTIO at 5, 7.5, 10, or 15 μM failed to block tetanus-induced potentiation (data not shown), whereas 30 μM carboxy-PTIO reliably blocked tetanus-induced potentiation without affecting basal synaptic transmission (see below).

The concentration chosen for the NOS inhibitor L-NAME was based on previous studies in which L-NAME attenuated tetanus-LTP under certain conditions (Williams et al., 1993a; Nicolarakis et al., 1994). L-NAME (100 μM, $n = 10$) was perfused for 60 min, and NO scavenger MGD-Fe (150/75 μM, $n = 7$) or carboxy-PTIO (30 μM, $n = 7$) was perfused for 30 min before tetanus (Fig. 2*A*). Drug containing ACSFs were switched to standard ACSF 5 min after tetanus. L-NAME, MGD-Fe, or carboxy-PTIO was applied separately. Both MGD-Fe and carboxy-PTIO significantly blocked tetanus-induced potentiation (111 ± 4 and 113 ± 13%, respectively, at 30 min after tetanus; Fig. 2*A,B*) compared with controls (standard ACSF, 189 ± 12%, at 30 min after tetanus; $n = 11$; $p < 0.05$). After perfusion of L-NAME for 60 min, there was a slight increase in field EPSP slopes (108 ± 5%; $n = 10$), which was not significantly different from the pretreatment baseline (104 ± 4% immediately before L-NAME perfusion). L-NAME attenuated tetanus-induced potentiation at 30 min after tetanus (146 ± 10%; $n = 10$) compared with controls ($p < 0.05$)

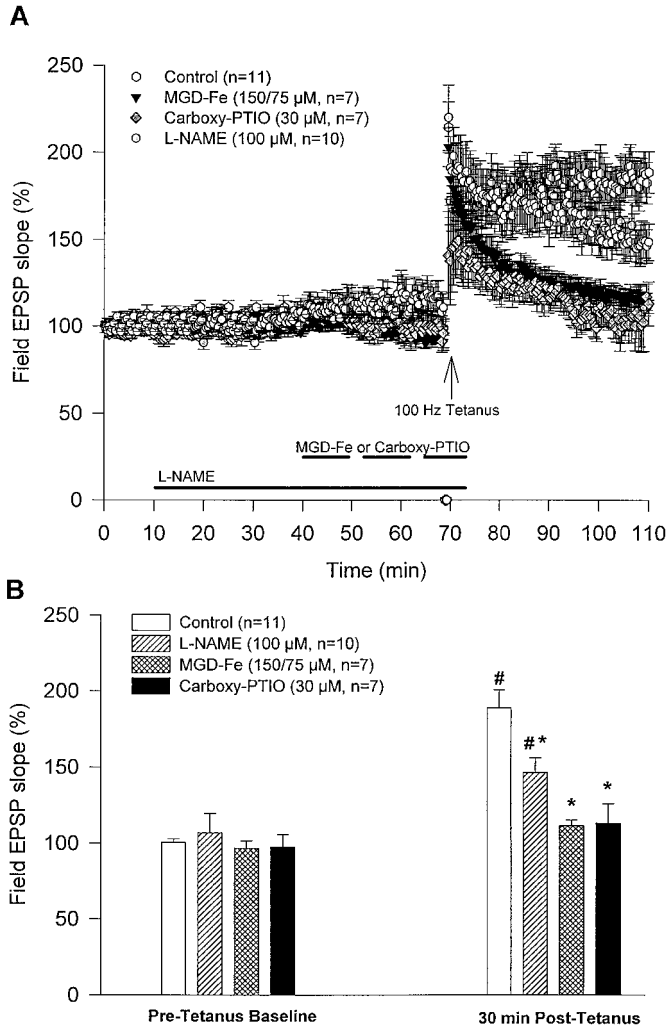

Figure 2. Extracellular perfusion of an NOS inhibitor (L-NAME) or NO scavengers (MGD-Fe and carboxy-PTIO) attenuate tetanus-induced synaptic potentiation. *A*, After stable baseline recordings, media containing the NOS inhibitor or NO scavengers were perfused for different durations (see Results) followed by tetanic stimulation. Five minutes after tetaus, perfusion media were switched to standard ACSF. Control slices ($n = 11$) were perfused with standard ACSF. *B*, L-NAME (100 μM; $n = 10$), MGD-Fe (150/75 μM; $n = 7$) or carboxy-PTIO (30 μM; $n = 7$) did not significantly affect basal synaptic transmission compared with controls. Thirty minutes after tetanus, synaptic potentiation was induced in both control and L-NAME groups, which was significantly different from the pretetanus baseline within the same group ($\#p < 0.05$). However, tetanus-induced potentiation in L-NAME, MGD-Fe, and carboxy-PTIO groups at 30 min after tetanus was significantly attenuated compared with controls ($^*p < 0.05$).

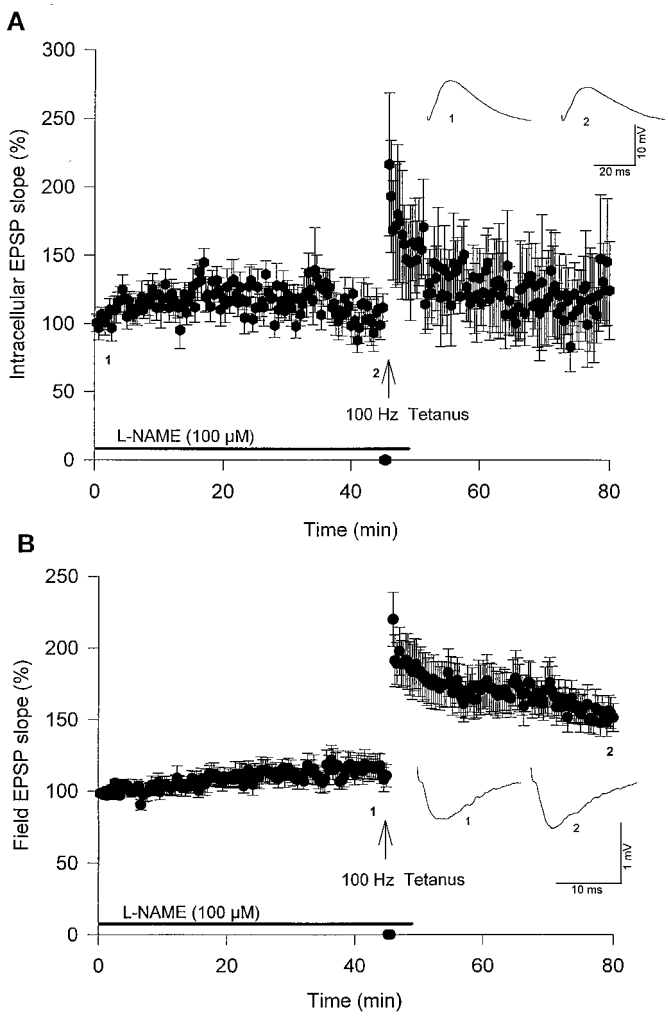

Figure 3. Extracellular L-NAME blocks Ca^{2+}/CaM-induced synaptic potentiation ($n = 10$). Slices were pretreated with L-NAME (100 μM) for $\geq$1 hr before beginning postsynaptic injections of Ca^{2+}/CaM. Five minutes after tetanus, slices were perfused with standard ACSF without L-NAME. *A*, Synaptic potentiation induced by postsynaptic Ca^{2+}/CaM injections was blocked by L-NAME. *B,* Simultaneous field recordings showed that tetanus-induced synaptic potentiation was attenuated by L-NAME.

and virtually blocked tetanus-induced potentiation when examined at 60 min after tetanus (113 ± 6%; no significant difference from the pretetanus baseline).

NOS inhibitor blocks Ca^{2+}/CaM-induced potentiation

To examine the possibility that NOS activity might contribute to synaptic potentiation induced by postsynaptic injection of Ca^{2+}/CaM, hippocampal slices were preincubated with L-NAME (100 μM) for 1 hr before being transferred to the recording chamber. Stable field recordings (field EPSPs) were established for at least 20 min, and then postsynaptic injections of Ca^{2+}/CaM with microelectrodes were performed. L-NAME (100 μM) was also present in the perfusate until 5 min after tetanus. Under these conditions, L-NAME significantly blocked Ca^{2+}/CaM-induced synaptic potentiation (104 ± 13%; $n = 10$, 45 min after beginning injections; Fig. 3*A*) compared with Ca^{2+}/CaM alone (184 ± 19%; $n = 5$, 45 min after beginning injections; Fig. 1*A*; $p < 0.05$). L-NAME also blocked tetanus-induced synaptic potentiation in Ca^{2+}/CaM-injected neurons (114 ± 29%; $n = 10$, 30 min after tetanus; Fig. 3*A*) compared with controls (at 30 min after tetanus; Fig. 1*C*; $p < 0.05$). In addition, L-NAME attenuated tetanus-induced potentiation of field EPSPs at 30 min after tetanus (138 ± 8%; $n = 10$; Fig. 3*B*) and blocked potentiation at 60 min after tetanus (115 ± 8%; $n = 10$; results not shown) compared with field EPSPs recorded during Ca^{2+}/CaM injections alone (148 ± 21%; $n = 5$, 60 min after tetanus; results not shown; $p < 0.05$).

To investigate whether postsynaptic NOS activity contributes to Ca^{2+}/CaM-induced synaptic potentiation, L-NAME was co-injected with Ca^{2+}/CaM into CA1 neurons. The addition of 100

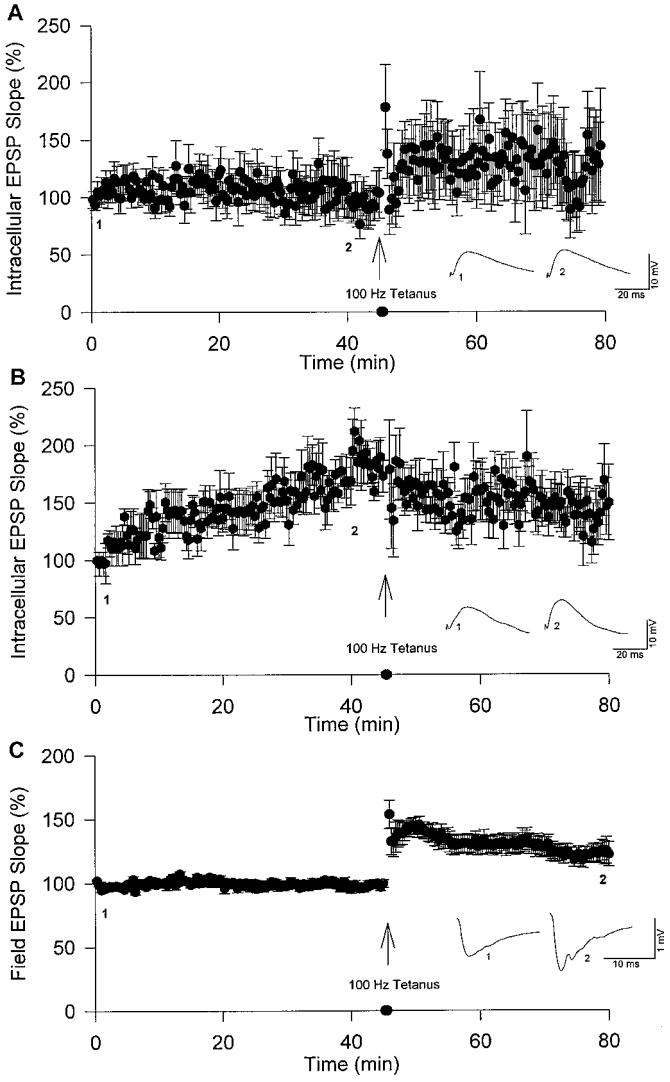

Figure 4. Postsynaptic co-injection of L-NAME, but not D-NAME, blocks Ca^{2+}/CaM-induced synaptic potentiation. *A,* Synaptic potentiation induced by postsynaptic co-injections of Ca^{2+}/CaM and L-NAME (100 mM; $n = 8$). *B,* Synaptic potentiation induced by postsynaptic co-injection of Ca^{2+}/CaM and D-NAME (67 or 100 mM; $n = 6$). *C,* Simultaneous field recordings showed synaptic potentiation induced by tetanus (L-NAME and D-NAME groups combined; $n = 14$).

mM L-NAME to Ca^{2+}/CaM (80/20 μM) in microelectrodes blocked Ca^{2+}/CaM-induced potentiation (98 ± 17%; $n = 8$, 45 min after beginning injections; Fig. 4*A*) compared with Ca^{2+}/CaM injections alone (184 ± 19%; $n = 5$, 45 min after beginning injections; Fig. 1*A*; $p < 0.05$). Co-injection of L-NAME and Ca^{2+}/CaM also blocked tetanus-induced synaptic potentiation (113 ± 30%; $n = 5$, 30 min after tetanus; Fig. 4*A*) compared with neurons injected with 2 M KAc alone (30 min after tetanus; Fig. 1C; $p < 0.05$). In contrast, co-injection of Ca^{2+}/CaM and D-NAME (67 or 100 mM), a stereoisomer much less potent than L-NAME, did not block Ca^{2+}/CaM-induced potentiation (178 ± 16%; $n = 6$, 45 min after beginning injections; Fig. 4*B*). Tetanic stimulation of neurons co-injected with D-NAME and Ca^{2+}/CaM resulted in depotentiation (Fig. 4*B*), which was consistent with previous results (Fig. 1*A*). Simultaneous extracellular field recordings in the same slices showed stable baselines for 45 min [99 ± 4%; $n = 14$ (L-NAME and D-NAME groups combined); Fig. 4*C*], and tetanus induced significant potentiation at 30 min (135 ± 7%; $n = 14$; Fig. 4*C*) or 60 min after tetanus (133 ± 10%; $n = 14$; results not shown) compared with pretetanus baseline ($p < 0.05$). Although L-NAME is a membrane-permeable NOS inhibitor, these results suggest that postsynaptic NOS activity is important for Ca^{2+}/CaM-induced synaptic potentiation.

Extracellular NO scavengers block tetanus-induced potentiation but not Ca^{2+}/CaM-induced potentiation

Since previous studies indicated that NO acted as a retrograde messenger at hippocampal synapses (O'Dell et al., 1991; Schuman and Madison, 1991, 1994a; Haley et al., 1992; Garthwaite and Boulton, 1995; Arancio et al., 1996), and our recent results showed the importance of NOS activity in Ca^{2+}/CaM-induced potentiation (see above; Ko and Kelly, 1998), we examined the role of NO as a retrograde messenger in Ca^{2+}/CaM-induced potentiation using extracellular applications of MGD-Fe or carboxy-PTIO to scavenge NO. Hippocampal slices were pretreated in the recording chamber with either MGD-Fe (150/75 μM; $n = 7$) for 60 min or carboxy-PTIO (30 μM; $n = 4$) for 30 min before obtaining extracellular recordings. Thirty minutes after establishing stable extracellular field recordings, postsynaptic injections of Ca^{2+}/CaM were initiated. MGD-Fe or carboxy-PTIO was present in the perfusate until 5 min after tetanus. Both MGD-Fe and carboxy-PTIO effectively blocked tetanus-induced synaptic potentiation (109 ± 7 and 115 ± 14%, respectively, 30 min after tetanus; Fig. 5*B,D*) compared with field recordings of Ca^{2+}/CaM injections alone (30 min after tetanus; Fig. 1*B*; $p < 0.05$). In contrast, extracellular perfusion of MGD-Fe or carboxy-PTIO did not block synaptic potentiation induced by postsynaptic injections of Ca^{2+}/CaM (170 ± 23 and 201 ± 44%, respectively, 45 min after beginning injections; Fig. 5*A,C*). Tetanic stimulation of Ca^{2+}/CaM-injected neurons treated with MGD-Fe or carboxy-PTIO resulted in depotentiation (Fig. 5*A,C*), which was consistent with previous results (Figs. 1*A*, 4*B*). These results suggest that NO does not act as a retrograde messenger in Ca^{2+}/CaM-induced synaptic potentiation, but NO still works as a retrograde messenger in tetanus-induced potentiation, which is consistent with previous reports (O'Dell et al., 1991; Schuman and Madison, 1991, 1994a; Haley et al., 1992; Arancio et al., 1996; Malen and Chapman, 1997).

Postsynaptic injection of NO scavenger blocks Ca^{2+}/CaM-induced potentiation

We have shown that NOS activity is essential for synaptic potentiation induced by postsynaptic injections of Ca^{2+}/CaM (Figs. 3*A,* 4*A*). On the other hand, extracellular administration of NO scavengers MGD-Fe or carboxy-PTIO did not block Ca^{2+}/CaM-induced potentiation (Fig. 5*A,C*). Taken together, these results suggest that NO produced by postsynaptic NOS, which is important for Ca^{2+}/CaM-induced potentiation, may not function as a retrograde messenger but acts directly at postsynaptic sites. To test this hypothesis, we co-injected Ca^{2+}/CaM and carboxy-PTIO (30 mM) into postsynaptic CA1 neurons. Co-injections of carboxy-PTIO significantly blocked Ca^{2+}/CaM-induced potentiation (120 ± 10%; $n = 6$, 45 min after beginning injections; Fig. 6*A*) compared with Ca^{2+}/CaM injections alone (45 min after beginning injections; Fig. 1*A*; $p < 0.05$). Postsynaptic injections of carboxy-PTIO alone (30 mM; Fig. 6*C*) did not affect basal synaptic transmission (114 ± 16%; $n = 4$, 45 min after beginning injections) but did block tetanus-induced potentiation (87 ± 10%

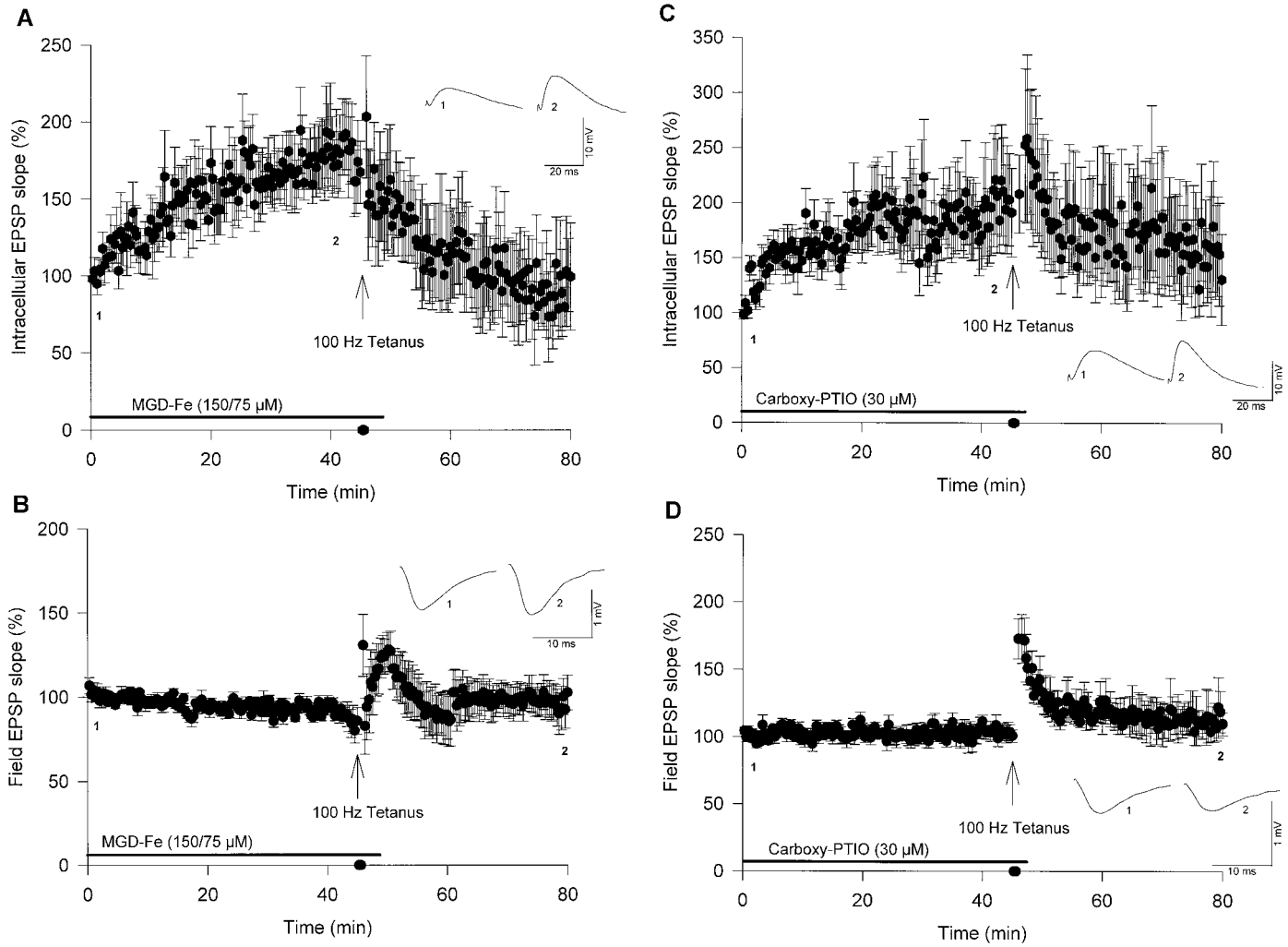

Figure 5. Extracellular applications of NO scavengers MGD-Fe and carboxy-PTIO block tetanus- but not Ca^{2+}/CaM-induced synaptic potentiation. *A, B*, Slices were pretreated with MGD-Fe (150/75 μM) for 30–60 min before postsynaptic injections of Ca^{2+}/CaM ($n = 7$). Five minutes after tetanus the medium was switched to standard ACSF. *A,* Synaptic potentiation induced by postsynaptic Ca^{2+}/CaM injections was not blocked by MGD-Fe. *B,* MGD-Fe blocked tetanus-induced synaptic potentiation in field recordings. *C, D*, Slices were pretreated with carboxy-PTIO (30 μM) for 30–60 min before postsynaptic injections of Ca^{2+}/CaM ($n = 4$). Three minutes after tetanus the medium was switched to standard ACSF. *C,* Synaptic potentiation induced by postsynaptic Ca^{2+}/CaM injections was not blocked by carboxy-PTIO. *D*, Carboxy-PTIO blocked tetanus-induced synaptic potentiation in field recordings.

at 30 min after tetanus). Simultaneous field recordings in these experiments showed stable baselines and tetanus-induced potentiation at 30 min after tetanus (145 ± 10 and 154 ± 14%, respectively; Fig. 6*B,D*) compared with their baseline values obtained 1–2 min before tetanus (both $p < 0.05$). These results indicate that NO acts as a postsynaptic and intracellular messenger during Ca^{2+}/CaM-induced synaptic potentiation but not as a retrograde messenger. On the other hand, NO functions, at least in part, as a retrograde messenger in tetanus-induced synaptic potentiation.

DISCUSSION

Our results show that postsynaptic injection of Ca^{2+}/CaM into hippocampal CA1 pyramidal neurons induces synaptic potentiation, which is consistent with previous reports (Wang and Kelly, 1995, 1996). In the nervous system, Ca^{2+}/CaM regulates many enzymes and channels (Rhoads and Friedberg, 1997), including adenylyl cyclase (Sunahara et al., 1996; Smit and Iyengar, 1998), Ca^{2+}/CaM-dependent protein kinases (Braun and Shulman, 1995), phosphodiesterases (Sharma, 1995; Zhao et al., 1997), NOS (Bredt and Snyder, 1994; Lee and Stull, 1998), calcineurin (Guerini, 1997; Klee et al., 1998), NMDA receptors (Ehlers et al., 1996; Hisatsune et al., 1997; Zhang et al., 1998), ryanodine receptors (Ikemoto et al., 1995; Guerrini et al., 1995), and cyclic nucleotide-gated channels (Molday, 1996; Zagotta and Siegelbaum, 1996). Many of these signaling molecules, including CaMKII and NOS, are believed to be involved in LTP (Malenka et al., 1989; Ocorr and Schulman, 1991; O'Dell et al., 1991; Schuman and Madison, 1991, 1994a,b; Lledo et al., 1995).

Postsynaptic injection of Ca^{2+}/CaM significantly decreases paired-pulse facilitation (PPF) (Wang and Kelly, 1996). Mechanisms responsible for changing PPF are believed to be presynaptic (Magleby, 1987; Zucker, 1989; but see Wang and Kelly, 1996, 1997b); therefore, changes in PPF are often interpreted to be attributable to presynaptic changes in transmitter release (Creager et al., 1980; Muller and Lynch, 1989; Manabe et al., 1993; Schulz et al., 1994). Postsynaptic injections of Ca^{2+}/CaM could

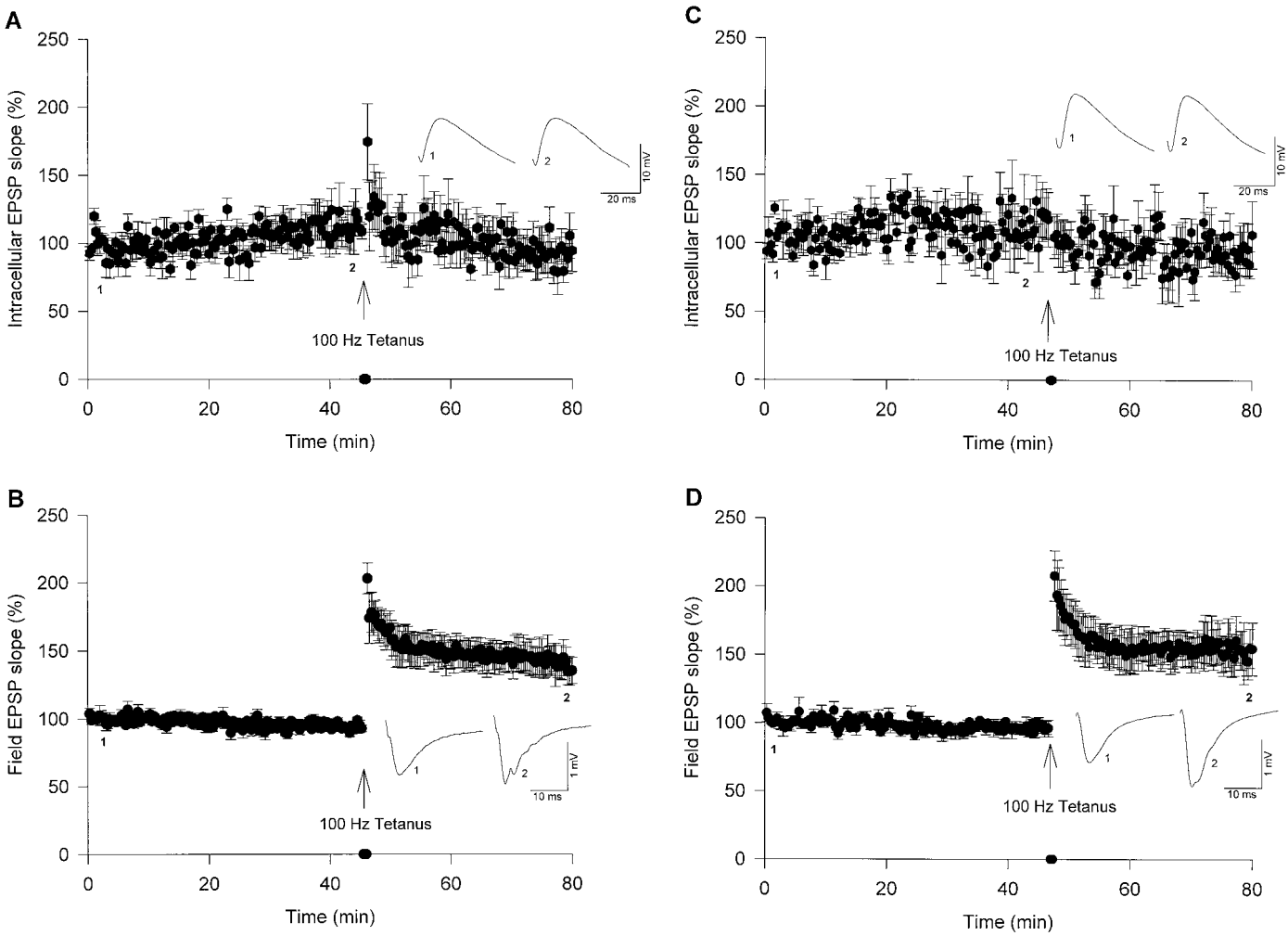

Figure 6. Postsynaptic injection of carboxy-PTIO blocks Ca^{2+}/CaM- and tetanus-induced synaptic potentiation. *A,* Postsynaptic co-injection of carboxy-PTIO (30 mM) blocked Ca^{2+}/CaM-induced synaptic potentiation ($n = 6$). *B,* Simultaneous field recordings showed synaptic potentiation induced by tetanus ($n = 6$). *C,* Postsynaptic injection of carboxy-PTIO (30 mM) alone blocked tetanus-induced synaptic potentiation ($n = 4$). *D,* Simultaneous field recordings showed synaptic potentiation induced by tetanus ($n = 4$).

activate NOS to produce NO (Bredt and Snyder, 1990, 1994; Bredt et al., 1992; Brenman et al., 1996), which may act like a retrograde messenger that contributes to PPF attenuation. NO is believed to function as an intercellular (retrograde) messenger in synaptic potentiation induced by tetanus or pairing protocols (O'Dell et al., 1991; Schuman and Madison, 1991, 1994a; Haley et al., 1992; Grathwaite and Boulton, 1995; Arancio et al., 1996; Malen and Chapman, 1997) (but see Chetkovitch et al., 1993; Williams et al., 1993a; Cummings et al., 1994).

Our results show that Ca^{2+}/CaM-induced potentiation can be blocked by the NOS inhibitor L-NAME through either extracellular perfusion or postsynaptic co-injection with Ca^{2+}/CaM. Thus, synaptic potentiation induced by postsynaptic injection of Ca^{2+}/CaM is NOS-dependent. To our surprise, extracellular perfusion of membrane-impermeable NO scavengers MGD-Fe and carboxy-PTIO did not decrease Ca^{2+}/CaM-induced potentiation, even though these NO scavengers strongly attenuated tetanus-induced potentiation. Therefore, NO appears not to be a retrograde messenger in Ca^{2+}/CaM-induced synaptic potentiation. In contrast, postsynaptic co-injection of carboxy-PTIO with Ca^{2+}/CaM blocked Ca^{2+}/CaM-induced synaptic potentiation. These results suggest that NO produced by the activation of NOS acts locally at a postsynaptic site(s) and is required during Ca^{2+}/CaM-induced synaptic potentiation.

Previous results indicate that NO might act at postsynaptic sites. First, NO-related species modulate NMDA receptor function by modulating its redox status and thereby decreasing Ca^{2+} influx (Lei et al., 1992; Lipton et al., 1993; Lipton and Wang, 1996; Lipton et al., 1996). In addition, Ca^{2+}/CaM can bind to NMDA receptors and reduce channel open probability (Ehlers et al., 1996). Thus, the dual actions of NO and Ca^{2+}/CaM on reducing NMDA-mediated Ca^{2+} influx could occur during Ca^{2+}/CaM-induced synaptic potentiation, suggesting that increased Ca^{2+} influx via NMDA receptors may not be important for this synaptic plasticity. Second, NO enhances Ca^{2+}/CaM-dependent phosphorylation of proteins in isolated postsynaptic density (PSD) fractions (Wu et al., 1996). The NO-stimulated enhancement of Ca^{2+}/CaM-dependent phosphorylation in PSDs may be important during Ca^{2+}/CaM-induced synaptic potentiation. Both CaMKII and AMPA receptors are present in hippocampal PSDs (Kelly et al., 1984, 1985; Riquelme et al., 1993; Rao et al., 1998). The apparent phosphorylation of AMPA receptors by CaMKII is enhanced during LTP (Barria et al., 1997), and this phosphorylation increases AMPA receptor conductance

(Derkach et al., 1998). Therefore, NO might increase the phosphorylation and conductance of AMPA receptors by CaMKII during Ca^{2+}/CaM-induced potentiation. Third, NO oxidizes neurogranin/RC3 (Mahoney et al., 1996; Sheu et al., 1996; Li et al., 1999). Neurogranin is a postsynaptic PKC substrate, which binds calmodulin in the absence of Ca^{2+} (Baudier et al., 1991; Huang et al., 1993; Gerendasy et al., 1995; Sato et al., 1995). Compared with reduced neurogranin, oxidized neurogranin binds CaM with lower affinity and is a poorer substrate for PKC (Mahoney et al., 1996; Sheu et al., 1996; Li et al., 1999). Thus, if neurogranin undergoes substantial NO-dependent oxidation during postsynaptic injections of Ca^{2+}/CaM, then more CaM and PKC could be available to enhance synaptic transmission.

Postsynaptic NO may activate additional signaling pathways, which could contribute to Ca^{2+}/CaM-induced synaptic potentiation. NO activates soluble guanylyl cyclase, which produces cGMP that then activates protein kinase G (PKG) (Garthwaite and Boulton, 1995). In rat hippocampus, the expression of guanylyl cyclase and CaM mRNAs are high in pyramidal neurons and dentate granule cells (Matsuoka et al., 1992). Thus, postsynaptic injections of Ca^{2+}/CaM could activate NOS and produce NO, which then stimulates guanylyl cyclase to enhance synaptic transmission through the activation of PKG. Inhibitors of guanylyl cyclase and PKG block the induction of LTP (Zhou et al., 1994a; Boulton et al., 1995), whereas cGMP analogs that activate PKG lower the threshold of LTP induction (Zhou et al., 1994b; Arancio et al., 1995). However, there is evidence that does not support a role of NO–cGMP–PKG pathways in synaptic potentiaton (Schuman et al., 1994; Selig et al., 1996; Wu et al., 1998; Kleppisch et al., 1999). Moreover, LTP is normal in mice lacking PKG-I and/or PKG-II but can be attenuated by an NOS inhibitor (Kleppisch et al., 1999). Thus, understanding the involvement of NO–cGMP–PKG signaling pathways in LTP and/or Ca^{2+}/CaM-induced potentiation awaits further investigation.

Another potential target for NO is ADP-ribosyltransferase (ADPRT; Schuman et al., 1994; Willmott et al., 1996). Extracellular application of ADPRT inhibitors block tetanus-induced potentiation (Schuman et al., 1994). Mice lacking PKG-I and/or -II display normal tetanus-LTP, but LTP is blocked by an ADPRT inhibitor (Kleppisch et al., 1999). NO can indirectly activate ADP-ribose cyclase to produce cyclic ADP-ribose (Willmott et al., 1996), which can enhance Ca^{2+} release from intracellular ryanodine-sensitive Ca^{2+} stores (Willmott et al., 1996). Calcium release from ryanodine-sensitive Ca^{2+} stores has been shown to contribute to tetanus-LTP in hippocampal slices (Obenaus et al., 1989; Wang et al., 1996; Wang and Kelly, 1997a). An additional intracellular target for NO is $p21^{ras}$ (Ras) (Yun et al., 1998). NO can activate immunoprecipitated neuronal Ras (Yun et al., 1998). Ras activation leads to phosphorylation and activation of mitogen-activated protein kinases (MAPKs), which regulate gene transcription and modulate long-term synaptic plasticity (Thomas et al., 1992; Wood et al., 1992; Moodie et al., 1993; Williams et al., 1993b; Yun et al., 1998). MAPKs are also involved in LTP induction in the hippocampal CA1 region (English and Sweatt, 1996, 1997). In summary, the ability of NO to modulate these additional pathways could contribute to Ca^{2+}/CaM-induced synaptic potentiation (Wang and Kelly, 1995).

Postsynaptic injections of Ca^{2+}/CaM (Wang and Kelly, 1995) or CaMKII (Lledo et al., 1995) induce synaptic potentiation. In both cases, potentiation induced by these postsynaptic manipulations occludes tetanus-LTP and vice versa (Lledo et al., 1995; Wang and Kelly, 1995, 1996). The expression of a constitutively active recombinant CaMKII in CA1 neurons potentiated synaptic transmission, which occluded tetanus-LTP (Pettit et al., 1994). Mutation studies with mice lacking α-CaMKII, or expressing an altered autophosphorylation phenotype of α-CaMKII indicated that CaMKII is required for tetanus-LTP (Silva et al., 1992; Giese et al., 1998). Ca^{2+}/CaM-induced potentiation and tetanus-LTP require postsynaptic Ca^{2+}/CaM-dependent protein kinase activities (Malenka et al., 1989; Malinow et al., 1989; Malinow and Tsien, 1990b; Lledo et al., 1995; Wang and Kelly, 1995). Thus, Ca^{2+}/CaM- and CaMKII-induced potentiation share common mechanisms with tetanus-LTP (Lledo et al., 1995; Wang and Kelly, 1995).

Here we report that even though both Ca^{2+}/CaM-induced potentiation and tetanus-LTP are NO-dependent, they are not the same. NO appears to function primarily as a retrograde messenger in LTP, because NOS inhibitors and extracellular NO scavengers block tetanus- or pairing-induced synaptic potentiation (O'Dell et al., 1991, 1994; Schuman and Madison, 1991, 1994a; Haley et al., 1992; Hawkins et al., 1994; Garthwaite and Boulton, 1995; Arancio et al., 1996; Malen and Chapman, 1997). However, these studies indicate that NO may also work at postsynaptic sites, because postsynaptic injections of a NOS inhibitor or NO scavenger blocked tetanus-LTP (Schuman and Madison, 1994a; Arancio et al., 1996). Similar to tetanus-LTP, Ca^{2+}/CaM-induced potentiation is blocked by extracellular application or postsynaptic co-injection of an NOS inhibitor. In contrast, Ca^{2+}/CaM-induced potentiation is blocked by postsynaptic co-injection of an NO scavenger, but not by extracellular applications of NO scavengers. Thus, we believe that NO acts at a postsynaptic site(s) during Ca^{2+}/CaM-induced potentiation. It is possible that during high-frequency stimulation or pairing protocols, presynaptic as well as postsynaptic components are activated through a variety of signal transduction cascades, so NO could react with its targets at both presynaptic and postsynaptic sites. Potentiation induced by injecting Ca^{2+}/CaM might activate postsynaptic NO targets without activating presynaptic targets. In conclusion, NO serves as a postsynaptic intracellular signaling molecule but not a retrograde messenger during Ca^{2+}/CaM-induced potentiation.

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