cAMP-Dependent Long-Term Potentiation of Nitric Oxide Release from Cerebellar Parallel Fibers in Rats

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Nitric Oxide (NO) is released from parallel fibers (PFs) after PF stimulation. NO–cGMP signaling is essential for long-term depression (LTD) in cerebellar PF–Purkinje cell synapses, which also exhibit presynaptic long-term potentiation (LTP) after tetanic PF stimulation. This LTP is dependent on cAMP but not NO–cGMP signaling. In this study, we analyzed long-term changes of NO release from PFs in rat cerebellar slices using electrochemical NO probes. Repetitive PF stimulation at 10 Hz for 2 sec elicited a transient increase in NO concentration (2.2 ± 0.1 nM; mean ± SEM; $n = 116$). This NO release exhibited long-term potentiation (LTP_{NO}) by 36 ± 3% ($n = 15$) after tetanic PF stimulation. Induction of LTP_{NO} was not affected by Glu receptor antagonists. NO release from PFs was also potentiated by L-Arg (ARG) (100 μM), forskolin (50 μM), and 8-bromo-cAMP (Br-cAMP) (1 mM) but not by 1,9-dideoxyforskolin (50 μM), a biologically inactive analog of forskolin. The potentiation induced by forskolin was significantly suppressed by H89 (10 μM), a blocker of cAMP-dependent protein kinase. The potentiation induced by forskolin, but not that induced by Arg, interfered with LTP_{NO}. H89 (10 μM) and KT5720 (1 μM), another blocker of cAMP-dependent protein kinase, but not KT5823 (300 nM), a blocker of cGMP-dependent protein kinase, significantly suppressed LTP_{NO}. These data indicate that neural NO release is under activity-dependent control, just as synaptic transmitter release is. LTP_{NO} might play a role in cross talk between presynaptic and postsynaptic plasticity by facilitating NO–cGMP-dependent postsynaptic LTD after induction of cAMP-dependent presynaptic LTP and LTP_{NO}.

Key words: nitric oxide; cAMP; cGMP; long-term potentiation; cerebellum; parallel fiber

Nitric oxide (NO) has various biological functions (Bredt and Snyder, 1994). Induction of long-term potentiation (LTP) in the hippocampal area CA1 is facilitated by NO (Böhme et al., 1991; Schuman and Madison, 1991; Arancio et al., 1996; Son et al., 1996), although the NO dependence is affected by various experimental conditions, such as temperature or animal age (Williams et al., 1993). Induction of LTP in layer II/III in the visual cortex does not depend on NO signaling (Kirkwood and Bear, 1994), whereas induction of LTP in layer V of the medial frontal cortex does (Nowicky and Bindman, 1993). Cerebellar long-term depression (LTD) in parallel fiber (PF)–Purkinje cell synapses is also dependent on NO–cGMP signaling for the induction (Crepel and Jaillard, 1990; Ito and Karachot, 1990; Shibuki and Okada, 1991; Daniel et al., 1993; Lev-Ram et al., 1995; Hartell, 1996a), whereas NO does not affect LTD of Glu-induced currents in cultured Purkinje cells (Linden et al., 1995) or LTD elicited by strong PF stimulation (Hartell, 1996b). Certain types of cerebellar motor learning, for which LTD is regarded as the cellular mechanism (Ito, 1989), are also dependent on NO signaling (Nagao and Ito, 1991; Li et al., 1995; Yanagihara and Kondo, 1996). These studies strongly suggest that NO is a modulator of synaptic plasticity.

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Of the isozymes of NO synthase (NOS), the neuronal type (nNOS) is widely distributed in the brain (Bredt et al., 1991). The nNOS activity is controlled by neural activities via Ca^{2+}–calmodulin (Bredt and Snyder, 1990). The nNOS molecule has several phosphorylation sites so that the function of nNOS may be modulated by phosphorylation (Brüne and Lapetina, 1991; Bredt et al., 1992). Although nNOS is a cytosolic enzyme, it has a high affinity for certain molecules, such as postsynaptic density proteins (Brenman et al., 1996a,b), and the intracellular distribution of nNOS may affect NO release. Activity-dependent changes in the distribution of nNOS immunoreactivity are found in the monkey visual cortex (Aoki et al., 1993). These data suggest the possibility that neural NO release may be under activity-dependent control. In our previous study, a slight potentiation of NO release has been found after tetanic PF stimulation (Shibuki and Kimura, 1997). The purpose of this paper is to study activity-dependent changes in NO release from PFs.

The mechanism of NO–cGMP-dependent LTD in PF–Purkinje cell synapses is explained by desensitization of postsynaptic Glu receptors (Ito et al., 1982; Linden et al., 1991; Nakazawa et al., 1995). Glu release from PFs, however, shows presynaptic LTP after tetanic PF stimulation (Salin et al., 1996; Linden, 1997), and this presynaptic LTP is dependent on cAMP but not cGMP (Salin et al., 1996). NO release in the molecular layer of the cerebellum is primarily derived from PFs, and the NO release is triggered by Ca^{2+} influx via voltage-gated Ca^{2+} channels (Shibuki and Kimura, 1997), just as Glu release from PFs is. Therefore, NO release from PFs may be modulated by cAMP. In this study, we investigate the roles of cyclic nucleotides in NO release from PFs.

MATERIALS AND METHODS

Slice preparations. Slices of the cerebellar vermis were prepared from Wistar rats of either sex (4–7 weeks old). The rat, anesthetized with ether, was immersed in ice-cold water, except for the nose, for 3 min to reduce brain temperature. Immediately after decapitation, the brain was removed, and coronal slices (400-μm-thick) of cerebellar vermis were prepared with a microslicer (DTK-2000; Dosaka, Osaka, Japan). The obtained slices were incubated in an artificial medium bubbled with 95% O_2 and 5% CO_2. The composition of the medium was (in mM): NaCl 124, KCl 5, NaH_2PO_4 1.24, $MgSO_4$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 26, and glucose 10, unless otherwise specified. When the Ca^{2+} concentration in the medium was changed, the sum of $MgSO_4$ and $CaCl_2$ concentrations was kept constant. After incubation at room temperature for more than 1 hr, the slices were transferred to a small recording chamber (~0.3 ml in volume) in which the slices were kept submerged. The recording chamber was maintained at 30°C and was continuously perfused with the oxygenated medium at the flow rate of 1 ml/min. The experiments were performed according to the guidelines of Niigata University and had been approved by the ethics committee of Niigata University.

Drugs. Forskolin was purchased from Wako Pure Chemical Industries (Osaka, Japan), and N^G-nitro-L-arginine (NA) was purchased from Sigma (St. Louis, MO). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), D-2-amino-5-phosphonovalerate (APV), and (RS)-α-methyl-4-carboxyphenylglycine (MCPG) were obtained from Tocris Cookson (Bristol, UK). H89 and KT5720 were obtained from Biomol Research Laboratory (Plymouth Meeting, MA), 1,9-dideoxyforskolin was from Research Biochemicals (Natick, USA), and KT5823 was from Kyowa Medex (Tokyo, Japan). These drugs were applied to the slices by adding into the perfusing medium.

NO probes. Electrochemical NO probes were fabricated as reported previously (Shibuki and Kimura, 1997). A glass pipette was obliquely polished such that the diameter of the tip was ~250 μm. After the edge was smoothed by flaming, the pipette was filled with a solution containing 30 mM NaCl and 0.3 mM HCl. The tip was sealed with a thin membrane of silicon rubber (TSE399; Toshiba). This membrane was prepared by placing a drop of TSE399 (~20–30 μl) on the surface of water. This drop spread over the surface of the water and polymerized within 50 min. The glass pipette was inserted slowly into the water through the silicon rubber membrane so that the tip of the pipette was sealed with the silicon rubber membrane. The shank of the pipette was painted with a small amount of TSE399 to ensure electrical insulation. The pipettes were left for several hours to allow hardening of the silicon rubber. As a working electrode, Teflon-coated Pt wire (metal diameter, 125 μm) was used. The tip was cut obliquely and heated in the tip of the flame of an ethyl alcohol lamp for a few seconds to remove the Teflon coating. The Pt wire, except at the tip, was insulated with heat-melted dental wax. The Pt wire was inserted into the pipette. The tip was protruded from the pipette (see Fig. 2*A*). A reference (Teflon-coated Ag wire) was also inserted into the pipette and was connected to the ground. The working Pt wire was connected to a current–voltage converter. The anode voltage between the working Pt wire and the reference Ag wire was maintained at +0.9 V. Each NO probe was calibrated by measuring the probe currents in response to a 30 μM NO solution, which was prepared by dissolving 36 μl of NO gas in 50 ml of degassed saline in a glass syringe. The sensitivity of the probes to NO was 0.2–1.1 nA/μM NO at 30°C at the anode voltage of +0.9 V, and the CO sensitivity under this condition was ~0.1% of the NO sensitivity. Usually, the NO probes could be used for more than a few weeks without significant changes in NO sensitivity.

Recording and stimulation. The PF volley potentials and field EPSPs elicited by PF stimulation were recorded extracellularly on the cut surface of the molecular layer in the slices through a glass micropipette filled with 2 M NaCl. The field potentials were elicited by single pulse stimulation at 12 sec intervals or by train pulse stimulation at 10 Hz for 2 sec. The five traces elicited by a single pulse or 20 traces elicited by 10 Hz train pulses were averaged to estimate the amplitude of the responses. The field EPSPs were isolated from the preceding PF volley potentials by subtracting the trace recorded in the presence of 10 μM CNQX from that recorded before CNQX application.

PFs were stimulated with biphasic pulses through the cut end of a Teflon-coated Ag wire placed on the surface of the slices. AgCl was deposited on the surface of the Ag wire by passing positive currents through the stimulating electrode in a NaCl solution. Every negative stimulus pulse was followed by a positive pulse, the absolute amplitude of which was 5% larger than that of the preceding negative pulse. Under these conditions, negative current from the stimulating electrode was mediated by Cl^- dissociated from AgCl, and generation of H_2 gas, which interfered with NO recording, was suppressed. The intensity of the negative stimulus pulse was 500 μA, unless otherwise specified. The duration of each pulse phase was 100 μsec. The tip of NO probe was placed on the surface of the molecular layer of cerebellar slices. The distance between the NO probe and the stimulating electrode was 100–200 μm. Although NO release elicited by PF stimulation with single pulse is not detected with NO probes, the NO release exhibits marked frequency facilitation (Shibuki and Kimura, 1997). Therefore, we used PF stimulation with pulse trains at 10 Hz for 2 sec for evoking NO release from PFs. The pulse trains were applied to slices at 2 min intervals. After the amplitude of NO release was stabilized, tetanic PF stimulation (TS) (5 pulses at 50 Hz, repeated at 2 sec intervals for 10 min) was applied to the slices. Because mild TS was not sufficient to potentiate NO release after the NO release elicited by 10 Hz stimulation was stabilized, we chose this prolonged tetanus protocol. To estimate the effect of TS on NO release, the amplitude of NO release was normalized by the averaged value in three consecutive traces recorded immediately before TS. The amplitude of potentiation of NO release was evaluated at 30 min after TS. Statistical significance between the data were evaluated by the Mann–Whitney *U* test, unless otherwise specified.

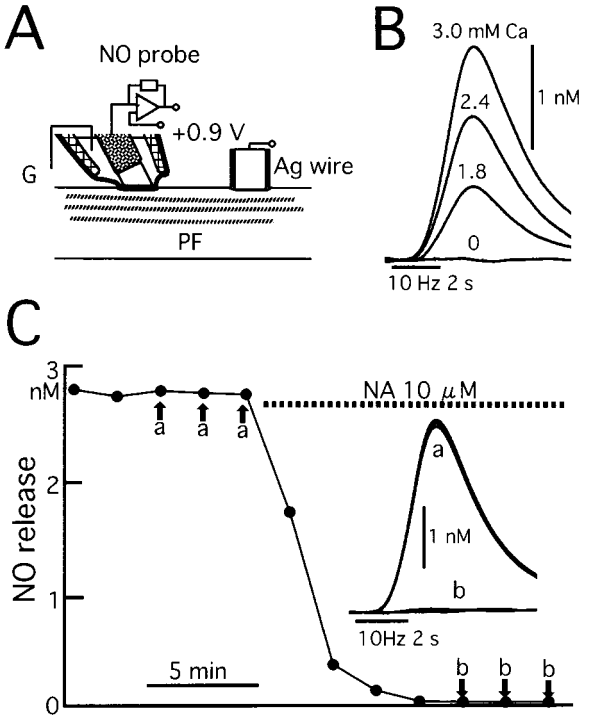

Figure 1. NO release from PFs in coronal cerebellar slices. *A*, Schema showing the experimental setup. *B*, NO release elicited by repetitive PF stimulation (10 Hz for 2 sec) and recorded under different extracellular Ca^{2+} concentrations (0–3.0 mM) in a slice. *C*, Amplitude of NO release elicited by repetitive PF stimulation at 2 min intervals and blockade of NO release by 10 μM NA. *Inset* shows superimposed original *traces* recorded before (*a*) and during (*b*) NA application.

RESULTS

NO recording in cerebellar slices

NO release was recorded in the molecular layer of coronal cerebellar slices using an electrochemical NO probe placed on the surface of the molecular layer (Fig. 1*A*). Repetitive PF stimulation (10 Hz for 2 sec) elicited a transient current increase in the NO probe (Fig. 1*B*). The current increase reached a peak between 3.0 and 4.2 sec after initiation of the PF stimulation (peak amplitude latency), and the time period during which the current change exceeded half the amplitude of the peak (half amplitude duration) was between 3.1 and 4.3 sec (n = 116). We varied

extracellular Ca^{2+} concentrations between 0 and 3.0 mM. Amplitude of the current in the NO probe was positively correlated with extracellular Ca^{2+} concentration (Fig. 1*B*). The current increase, which corresponded to 0.7–4.6 nM NO (2.2 ± 0.1 nM; mean ± SEM; $n = 116$), was completely blocked by the addition of 10 μM NA, a specific NOS blocker, into the medium perfusing the slice (Fig. 1*C*). These data indicate that NO release from PFs was reflected in the increase of current through the NO probe, as reported previously (Shibuki and Kimura, 1997).

LTP of NO release from PFs

NO release elicited by repetitive PF stimulation exhibits gradual potentiation for 30–50 min after initiation of NO recording (Shibuki and Kimura, 1997). In this study, we observed similar gradual potentiation after initiation of NO recording (Fig. 2*A*). TS (5 pulses at 50 Hz, repeated at 2 sec interval for 10 min) was applied to the slices after the amplitude of NO release was stabilized. Potentiation of NO release was elicited by TS (Fig. 2*A*). The maximal amplitude of this potentiation (67 ± 7%; $n = 15$) was observed within a few minutes after cessation of TS. Subsequently, amplitude of NO release was gradually reduced. However, the potentiation lasted for >30 min (Fig. 2*B*), and the amplitude of potentiation 30 min after cessation of TS was 36 ± 3% ($n = 15$). Neither the peak amplitude latency nor half amplitude duration was changed by TS (Fig. 2*A*, *inset*). In the absence of TS, no clear potentiation of NO release was observed (Fig. 2*B*,*a*). PF volley potentials elicited by a single pulse ($n = 5$) (Fig. 2*B*,*b*) or the averaged potentials of 20 traces elicited by PF stimulation at 10 Hz for 2 sec ($n = 5$) (Fig. 2*B*,*c*) exhibited no potentiation after TS. These data strongly suggest that LTP_{NO} was elicited by TS.

Presynaptic LTP of Glu release is also elicited by TS (Salin et al., 1996). Therefore, field EPSPs in Purkinje cells and NO release were simultaneously recorded at the both sides of single stimulating electrode in five slices (Fig. 2*C*, *inset*). During the initial 50 min of recording, NO release was gradually potentiated by 28 ± 6% ($n = 5$), whereas the averaged field EPSPs of 20 traces elicited by PF stimulation at 10 Hz for 2 sec exhibited only modest potentiation (8 ± 9%; $n = 5$) (Fig. 2*C*). TS elicited LTP_{NO} by 38 ± 7% in the five slices, whereas no clear potentiation of the averaged field EPSPs were observed after TS (Fig. 2*C*). The difference in the TS-induced potentiation between NO release and averaged field EPSPs was significant ($p < 0.05$; Wilcoxon signed rank test). Similar discrepancy between NO release and field EPSPs was also found in the experiments in which the stimulus intensity was decreased from 500 to 400 μA ($n = 5$), or the Ca^{2+} concentration in the perfusing medium was decreased from 2.4 to 1.8 mM ($n = 5$) (Fig. 2*C*). The reduction of NO release was significantly larger than that of the field EPSPs in these experiments ($p < 0.05$ for both experiments; Wilcoxon signed rank test). These results strongly suggest that field EPSPs were saturated under the experimental conditions used for recording LTP_{NO}. Therefore, we reduced the intensity of PF stimulation from 500 to 50 μA so that only field EPSPs were recorded clearly (data not shown). However, the field EPSPs elicited by test stimuli of 10 Hz for 2 sec exhibited almost no change after TS at the stimulus intensity of 50 μA, and the amplitude of the averaged field EPSPs 30 min after cessation of TS was 99 ± 3% ($n = 5$) of that recorded immediately before TS.

In the molecular layer, PFs make synapses with neurons, among which basket neurons express nNOS (Bredt et al., 1991). To ascertain the possible contribution of postsynaptic neurons of PFs

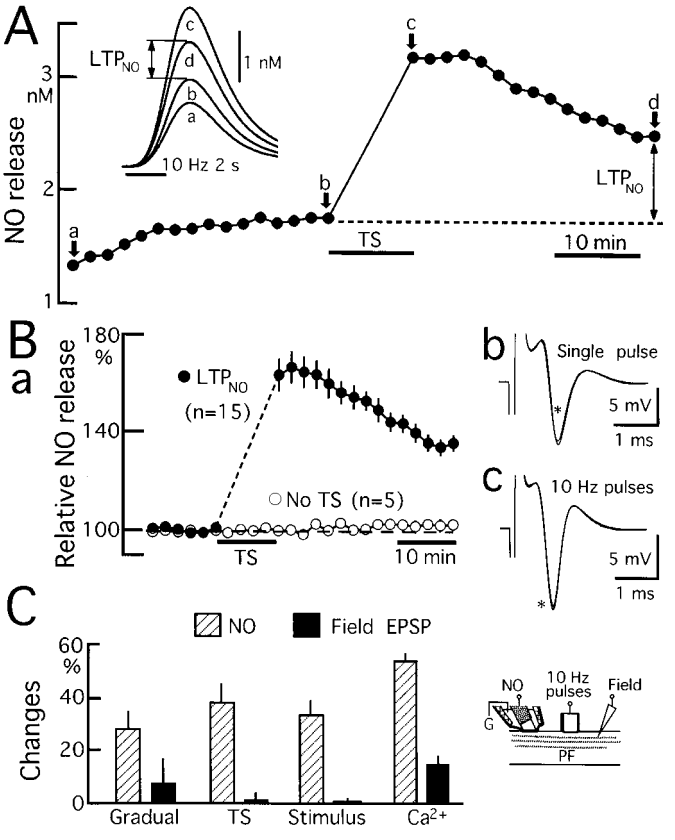

Figure 2. LTP_{NO} elicited by TS. *A*, Amplitude of NO release recorded before and after TS. *Inset* shows superimposed *traces* of NO release recorded at the initiation of recording (*a*), immediately before initiation of TS (*b*), immediately after cessation of TS (*c*), and 30 min after cessation of TS (*d*). *B*,*a*, Time course of relative NO release before and after TS (*filled circles*) or in the absence of TS (*open circles*). *B*,*b*, Averaged PF volley potentials elicited by single pulse stimulation immediately before initiation and 30 min after cessation of TS (*asterisk*). *B*,*c*, Averaged PF volley potentials elicited by 10 Hz train pulses immediately before initiation and 30 min after cessation of TS (*asterisk*). *C*, Changes in NO release (*hatched bars*) and field EPSPs (*filled bars*). Gradual potentiation during the initial 50 min of the recording (*Gradual*), TS-induced potentiation (*TS*), the reduction caused by the change in stimulus intensity from 500 to 400 μA (*Stimulus*), and the reduction caused by the change in extracellular Ca^{2+} concentration from 2.4 to 1.8 mM (*Ca2+*) are shown. The amplitude of each change was normalized by the amplitude of NO release or field EPSPs recorded before the change occurred. Each *bar* and *error bar* represent the absolute value of the mean ± SEM of five experiments. Except for the experiment in which extracellular Ca^{2+} concentration was decreased, NO release and field EPSPs were simultaneously recorded in the same slice, as shown by the *inset*.

to LTP_{NO}, we applied CNQX (10 μM), an antagonist of non-NMDA receptors. However, amplitude of NO release was not clearly affected by CNQX (Fig. 3*A*), suggesting that most of the NO was derived from PFs. Furthermore, LTP_{NO} was elicited by TS in the presence of 10 μM CNQX. Although LTP_{NO} was slightly reduced in the presence of 10 μM CNQX, no significant difference was found in the LTP_{NO} amplitude elicited in the absence or presence of 10 μM CNQX (Fig. 3*D*). We studied the contribution of other Glu receptors to the induction of LTP_{NO}. However, LTP_{NO} amplitude was significantly affected by neither APV (50 μM), a blocker of NMDA receptors, nor MCPG (500 μM), a blocker of metabotropic Glu receptors (Fig. 3*B–D*). Simultaneous application of CNQX and MCPG, both of which

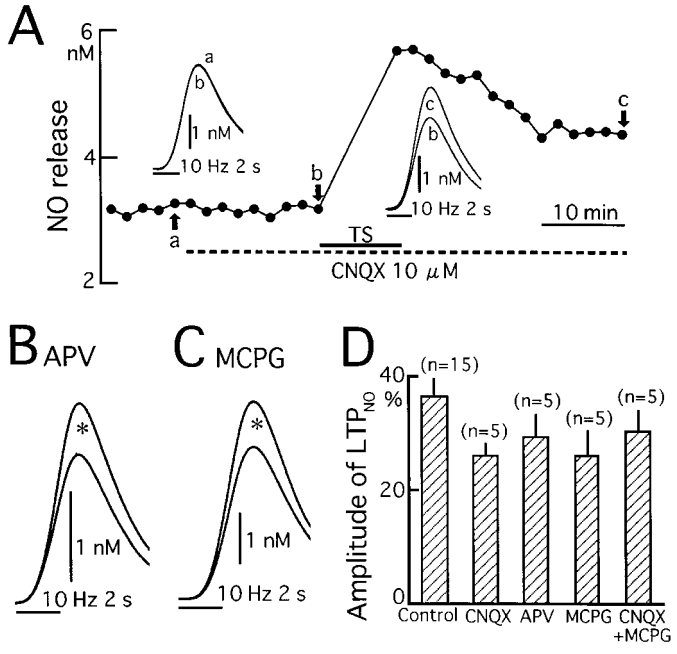

Figure 3. Effects of Glu blockers on LTP_{NO}. *A*, Amplitude of NO release recorded before, during, and after application of CNQX (10 μM) and TS. *Insets* show superimposed *traces* recorded immediately before application of CNQX (*a*) or TS (*b*) and 30 min after cessation of TS (*c*). *B*, Superimposed *traces* recorded before and 30 min after TS (*asterisk*), which was applied to the slice in the presence of 50 μM APV. *C*, *Traces* recorded before and 30 min after TS (*asterisk*) applied in the presence of 500 μM MCPG. *D*, Amplitude of control LTP_{NO} and LTP_{NO} elicited in the presence of 10 μM CNQX, 50 μM APV, 500 μM MCPG, or 10 μM CNQX plus 500 μM MCPG. The mean ± SEM are shown.

block postsynaptic Glu receptors in Purkinje cells, was also ineffective (Fig. 3*D*).

NO is produced from L-arginine (Arg). NO release from PFs was augmented by the addition of Arg into the perfusing medium in a dose-dependent manner (Fig. 4*A*). The augmentation of the NO release by Arg reached a plateau at an Arg concentration of 100 μM (Fig. 4*A*). Because this augmentation by 100 μM Arg (41 ± 8%; $n = 5$) is comparable with the LTP_{NO} amplitude, the mechanism of LTP_{NO} might involve augmentation of Arg supply to PFs. To investigate this possibility, induction of LTP_{NO} was tested in the presence of 100 μM Arg (Fig. 4*B*,*C*). However, the LTP_{NO} amplitude (35 ± 4%; $n = 5$) was not significantly different from that in normal medium (Fig. 4*C*), suggesting that the mechanism of LTP_{NO} probably does not involve augmentation of Arg supply to PFs.

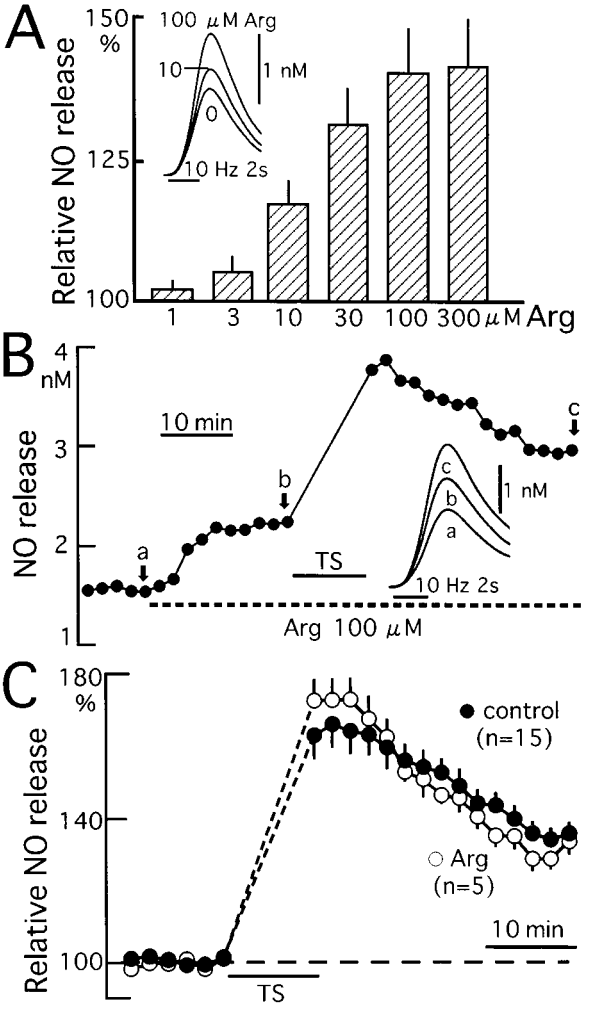

Figure 4. LTP_{NO} in the presence of excessive Arg. *A*, Dependence of the amplitude of NO release on Arg concentration in the perfusing media. Data represent the mean ± SEM of five experiments. The values are normalized by that recorded in the perfusing medium not containing Arg. *Inset* shows superimposed *traces* recorded in a slice in perfusing media containing 0–100 μM Arg. *B*, Changes in amplitude of NO release elicited by 100 μM Arg application and TS. *Inset* shows superimposed *traces* recorded immediately before Arg application (*a*), immediately before initiation (*b*), and 30 min after cessation of TS (*c*). *C*, Time courses of LTP_{NO} in the presence (*open circles*) or absence of 100 μM Arg (*filled circles*).

Dependence of the induction of LTP_{NO} on cAMP

Presynaptic LTP of Glu release from PFs is dependent on cAMP (Salin et al., 1996). Therefore, we studied the effect of forskolin, an activator of adenylate cyclase, on NO release from PFs. NO release exhibited potentiation after the addition of 50 μM forskolin into the perfusing medium (Fig. 5*A*). Potentiation of NO release occurred gradually during forskolin application of 50 min. The maximal amplitude of the potentiation was 62 ± 17% ($n = 7$), which is comparable with the maximal amplitude of the potentiation elicited by TS. The forskolin-induced potentiation continued even after cessation of forskolin application, and the amplitude of the potentiation 30 min after cessation of forskolin application was 56 ± 18% ($n = 7$). The corresponding potentiation elicited by forskolin was 10 ± 12% ($n = 5$) in the presence of 10 μM H89, a blocker of cAMP-dependent protein kinase, and this value was significantly smaller than that in normal medium ($p < 0.05$) (Fig. 5*A*). Furthermore, the amplitude of the potentiation observed 30 min after cessation of the application of 1,9-dideoxyforskolin (50 μM for 50 min), a biologically inactive forskolin analog, was only 1 ± 4% ($n = 5$) (Fig. 5*B*) and was significantly smaller than that observed after forskolin application ($p < 0.001$). These results strongly suggest that the forskolin-induced potentiation of NO release is mediated by cAMP.

We speculated that forskolin-induced potentiation of NO release may share the mechanism with that of LTP_{NO} elicited by TS. To investigate this possibility, TS was applied to the slices during forskolin application. However, TS elicited only transient potentiation of NO release (Fig. 5*B*), and the maximal amplitude of the potentiation elicited by TS during forskolin application

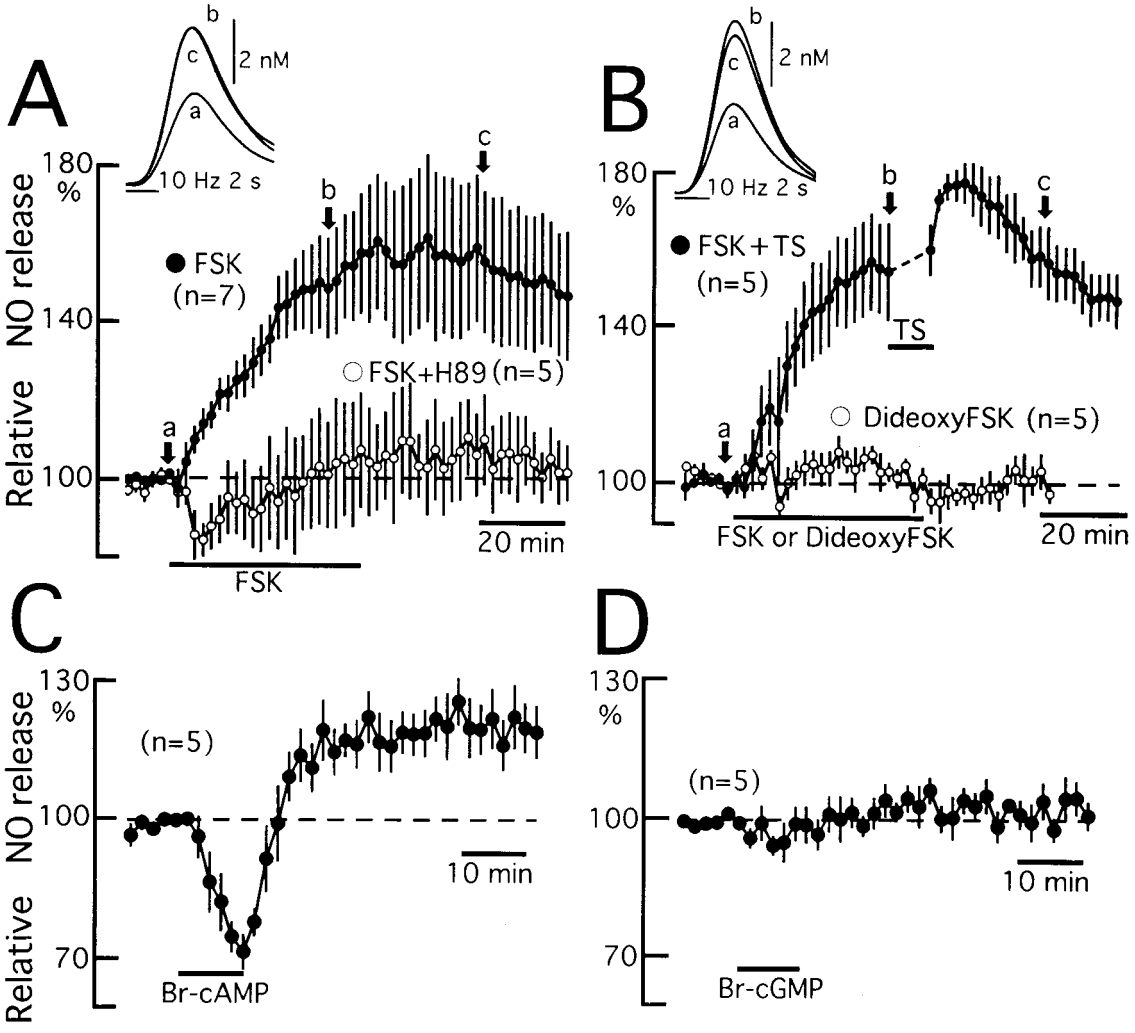

Figure 5. Potentiation of NO release induced by forskolin (*FSK*) and a cAMP analog. *A*, Potentiation of NO release induced by 50 μM forskolin (*filled circles*). *Inset* shows superposed *traces* recorded before application of forskolin (*a*), in the presence of forskolin (*b*), and 30 min after the cessation of forskolin application (*c*). Forskolin-induced potentiation in the presence of 10 μM H89 is also shown (*open circles*). In this experiment, slices were incubated with 10 μM H89 for at least 2 hr before and throughout the recording. *B*, Potentiation of NO release induced by forskolin and TS (*filled circles*). *Inset* shows *traces* recorded before application of forskolin (*a*), before initiation of TS (*b*), and 30 min after the cessation of forskolin application and TS (*c*). Changes in NO release elicited by application of 1,9-dideoxyforskolin (50 μM, *DideoxyFSK*) are also shown (*open circles*). *C*, Potentiation of NO release induced by 1 mM Br-cAMP (*horizontal bar*). *D*, Effect of 1 mM Br-cGMP (*horizontal bar*) on NO release.

(78 ± 5%; $n = 5$) was comparable with that elicited by TS alone (67 ± 7%; $n = 15$). Furthermore, the amplitude of the potentiation 30 min after cessation of forskolin application and TS together (57 ± 9%; $n = 5$) was not significantly different from the amplitude of the potentiation elicited by forskolin alone (56 ± 18%; $n = 7$). These findings are well explained if forskolin-induced potentiation and TS-induced LTP_{NO} share the same molecular mechanism.

To study the relationship between NO release and cAMP further, we applied 8-bromo-cAMP (Br-cAMP), a membrane-permeable analog of cAMP, to the slices (Fig. 5*C*). During the application of 1 mM Br-cAMP, NO release was reduced by 28 ± 4% ($n = 5$). However, NO release recovered after washing out the Br-cAMP, and substantial potentiation of NO release (19 ± 5%) was observed 30 min after cessation of Br-cAMP application (Fig. 5*C*). In contrast, 8-bromo-cGMP (Br-cGMP) (1 mM), a membrane-permeable analog of cGMP, exhibited almost no effect on NO release (Fig. 5*D*), and there was a significant difference in the amplitude of potentiation elicited by Br-cAMP and Br-cGMP ($p < 0.03$).

The facilitatory effect of cAMP on the induction of presynaptic LTP is blocked by H89 or KT5720, another blocker of A kinase A (Weisskopf et al., 1994; Salin et al., 1996). Therefore, we studied LTP_{NO} in slices incubated with 10 μM H89 or 1 μM KT5720. Although TS elicited transient potentiation of NO release, amplitude of the potentiation 30 min after cessation of TS was 2 ± 3% ($n = 5$) in the presence of 10 μM H89 and 12 ± 3% ($n = 5$) in the presence of 1 μM KT5720 (Fig. 6*A*). These values were significantly smaller than those recorded in slices incubated in normal medium ($p < 0.005$ for both data). As control, we studied LTP_{NO} in the slices incubated with 300 nM KT5823, a specific blocker of G kinase and NO–cGMP-dependent LTD (Ito and Karachot, 1992; Hartell, 1996a). However, no apparent effect of KT5823 on LTP_{NO} was found (Fig. 6*A*). The amplitude of LTP_{NO} elicited by TS in the presence of KT5823 (36 ± 4%; $n = 5$) was not significantly different from that of LTP_{NO} elicited by

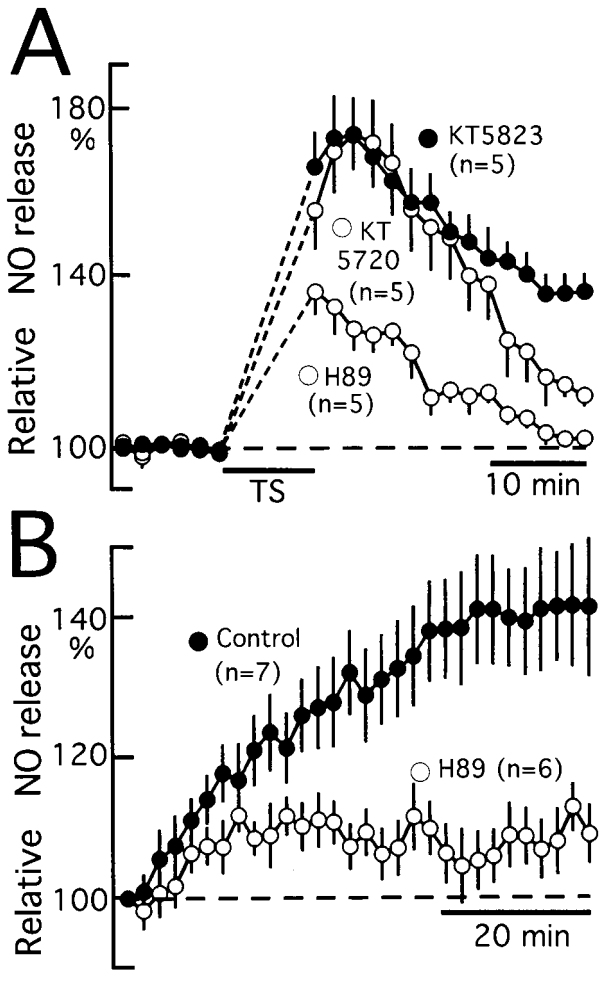

Figure 6. Blockade of LTP_{NO} and gradual potentiation of NO release by H89 or KT5720. *A*, Time course of LTP_{NO} recorded in the presence of 10 μM H89 (*open circles*), 1 μM KT5720 (*open circles*), or 300 nM KT5823 (*filled circles*). *B*, Time courses of gradual potentiation of NO release induced by test stimuli in the absence (*filled circles*) or presence of 10 μM H89 (*open circles*). Slices were incubated with 10 μM H89, 1 μM KT5720, or 300 nM KT5823 for at least 2 hr before and throughout the recording.

TS in normal medium. These results, together with the data obtained from the forskolin and Br-cAMP experiments, indicate that LTP_{NO} is dependent on cAMP but not on cGMP.

Effect of H89 on the gradual potentiation of NO release

NO release from PFs exhibits marked frequency facilitation (Shibuki and Kimura, 1997). In this study, we adopted PF stimulation of 10 Hz pulse trains to elicit clear NO release for quantitative analysis. Because repetitive PF stimulation at 8 Hz is sufficient to elicit LTP of Glu release from PFs (Salin et al., 1996), we expected that PF stimulation at 10 Hz may be sufficient to elicit potentiation of NO release from PFs. In accordance with this expectation, we observed the gradual potentiation during a period of 30–50 min after initiation of NO recording (Figs. 2*A*, 6*B*). We recorded the gradual potentiation in the slices incubated with 10 μM H89 (Fig. 6*B*). The amplitude of the gradual potentiation during the initial 1 hr of NO recording was 9 ± 4% ($n = 6$), and this value was significantly smaller than that recorded in normal medium (43 ± 10%; $n = 7$; $p < 0.02$). This result suggests that repetitive PF stimulation at 10 Hz is sufficient to elicit potentiation of NO release in a cAMP-dependent manner.

DISCUSSION

LTP of NO release from PFs

In this study, we used an electrochemical NO probe whose tip was covered by a thin rubber membrane (Shibuki, 1990; Shibuki and Okada, 1991; Shibuki and Kimura, 1997). Because neither current nor electrolytes are released from the tip of the probe to the surrounding space, NO recording using this NO probe is suitable for detecting long-term changes in NO release without damaging tissue. In our previous study, we detected NO release from PFs elicited by PF stimulation using this probe (Shibuki and Kimura, 1997). The release of NO was identified by the characteristic dependency of the responses on the anode voltage in the probe and the sensitivity to NA. Furthermore, we found that NO release from PFs elicited by 20 Hz PF stimulation was slightly potentiated after TS (Shibuki and Kimura, 1997). However, the amplitude of the potentiation 30 min after cessation of TS was only ~10%, and therefore further analysis was not practicable. In this study, we adopted 10 Hz PF stimulation as test stimuli, and the amplitude of potentiation 30 min after cessation of TS was increased to 36 ± 3% ($n = 15$). This potentiation is not attributed to changes in the sensitivity of the NO probe, which usually showed stability for more than a few weeks. The probe currents elicited by PF stimulation were completely blocked by 10 μM NA, even after TS (data not shown), and therefore the changes in the probe current must reflect net changes in NO concentration. Suppression of degradation of NO is unlikely to be caused by TS, because the time course of the probe currents was not changed by TS (Fig. 2*A*, *inset*). No clear potentiation of NO release was observed in the absence of TS (Fig. 2*B*,*a*). Amplitude of PF volley potentials was not potentiated by TS (Fig. 2*B*,*b*), and therefore changes in tissue excitability cannot explain the potentiation of the probe current. From these results, it is concluded that LTP_{NO} is elicited by TS in cerebellar slices.

Comparison between LTP_{NO} and cAMP-dependent presynaptic LTP

Although NO release is not dependent on exocytosis, Ca^{2+} influx via voltage-gated Ca^{2+} channels triggers not only Glu release but also NO release from PFs. Therefore, comparison of NO release and EPSPs in Purkinje cells may be of interest. The filed EPSPs simultaneously recorded with LTP_{NO} exhibited no clear potentiation after TS. This result and the similar discrepancy regarding the dependency on extracellular Ca^{2+} concentration or stimulus intensity indicate that field EPSPs were saturated at the stimulus intensity of 500 μA, which was used for recording LTP_{NO}. The amplitude of PF volley potentials is almost linearly correlated to the stimulus intensity up to 500 μA under our experimental conditions (Shibuki and Kimura, 1997). Therefore, the saturation of field EPSPs at 500 μA cannot be attributable to saturated PF excitation but is probably explained by saturated postsynaptic depolarization. At the stimulus intensity of 50 μA, however, field EPSPs elicited by test stimuli of 10 Hz for 2 sec exhibited almost no change after TS, suggesting that LTP_{NO} and LTP of field EPSP may not necessarily occur in parallel.

In the hippocampus, there are two typical types of LTP (Bliss and Collingridge, 1993; Nicoll and Malenka, 1995). LTP in CA1 pyramidal neurons requires postsynaptic Ca^{2+} rise for the induction, whereas presynaptic LTP in mossy fiber–CA3 pyramidal neuron synapses does not require synaptic transmission and postsynaptic increase in Ca^{2+} concentration for the induction (Castillo et al., 1994; Weisskopf et al., 1994). Presynaptic LTP in sympathetic ganglion synapses (Kuba and Kumamoto, 1986), the

mossy fiber synapses (Weisskopf et al., 1994), and cerebellar PF–Purkinje cell synapses (Salin et al., 1996) are dependent on cAMP for induction. LTP_{NO} was elicited in the presence of Glu receptor antagonists (Fig. 3) and is unlikely to be dependent on increase in Ca^{2+} concentration in postsynaptic neurons of PFs. The induction of LTP_{NO} was dependent on cAMP and activation of kinase A (Figs. 5, 6). These characteristics of LTP_{NO} are similar to those of cAMP-dependent presynaptic LTP.

Possible molecular mechanism for LTP_{NO}

Electrical white matter stimulation causes an increase in the Arg level in rat cerebellar slices (Hansel et al., 1992). In the molecular layer, Arg is predominantly localized in Bergmann glial cells (Aoki et al., 1991). Glial synaptic currents exhibit LTP after repetitive stimulation of granule cells (Linden, 1997). Application of Arg to the slices potentiated NO release from PFs (Fig. 4*A*). Therefore, activity-dependent changes in Arg supply might be responsible for LTP_{NO} elicited by TS. However, this hypothesis does not seem likely, because the amplitude of LTP_{NO} elicited in the presence of excess Arg was not significantly different from that of LTP_{NO} elicited in normal medium (Fig. 4*C*). The primary structure of nNOS has several phosphorylation sites that are recognized by protein kinases (Bredt et al., 1992). However, activation of A or C kinases does not potentiate nNOS activity (Brüne and Lapetina, 1991; Bredt et al., 1992). Therefore, upregulation of Arg supply or potentiation of nNOS activity by phosphorylation does not seem to be responsible for the induction of LTP_{NO} elicited by TS.

NO release from PFs is triggered by Ca^{2+} influx (Shibuki and Kimura, 1997), and NO release was positively correlated with extracellular Ca^{2+} concentration (Fig. 1*B*). Therefore, upregulation of Ca^{2+} influx is a possible mechanism for LTP_{NO}. Presynaptic LTP in the mossy fiber synapses interferes with paired pulse facilitation or frequency facilitation of synaptic transmission (Weisskopf et al., 1994). However, presynaptic LTP, but not paired pulse facilitation or frequency facilitation, is impaired in the Rab 3A deficient mice (Castillo et al., 1997). Rab 3A is a small G-protein involved in exocytosis of synaptic vesicles (Geppert et al., 1994, 1997). Therefore, changes in Ca^{2+} influx may not be responsible for cAMP-dependent presynaptic LTP, although Ca^{2+} influx is required for the induction (Castillo et al., 1994). Because NO release from PFs is also triggered by presynaptic Ca^{2+} influx (Shibuki and Kimura, 1997), changes in Ca^{2+} influx might not be responsible for LTP_{NO}.

Although nNOS is a cytosolic enzyme, it has affinity for specific molecules, such as postsynaptic density (PSD) proteins (Brenman et al., 1996a,b). In the monkey visual cortex, monocular deprivation elicits a decrease in nNOS immunoreactivity in the axon terminals in the corresponding cortical columns (Aoki et al., 1993). Because PSD proteins are also found in the cerebellum (Brenman et al., 1996a,b), distribution of nNOS molecules might be inhomogeneous in granule cells, and activity-dependent changes in nNOS distribution in granule cells might be elicited by TS. It has been proposed that exocytotic fusion is elicited by a very large and localized increase in Ca^{2+} concentration near the Ca^{2+} channels immediately after each action potential (Simon and Llinás, 1985). The NO release from PFs was sensitively dependent on extracellular Ca^{2+} concentration (Fig. 1*B*), and therefore even a slight change in the distribution of nNOS molecules with respect to the location of voltage-gated Ca^{2+} channels in PFs might be sufficient to modify NO release. However, no direct evidence supporting this presumption is available at present. Transmitter release is dependent on the complex mechanisms, including Ca^{2+}-dependent exocytosis, whereas NO release from PFs is elicited simply by activation of nNOS via Ca^{2+}–calmodulin. Therefore, understanding the mechanism for LTP_{NO} might help the elucidation of the mechanism for presynaptic plasticity.

Physiological roles of LTP_{NO}

Although we used TS with 50 Hz pulses for induction of LTP_{NO}, gradual potentiation of NO release, which probably shares a molecular basis with LTP_{NO}, was elicited by 10 Hz PF stimulation. Because granule cells can fire at high frequency *in vivo* (Eccles et al., 1966), LTP_{NO} may be elicited *in vivo*. What is the physiological role of LTP_{NO} *in vivo*? Cerebellar LTD in PFs–Purkinje cell synapses is elicited by conjunctive stimulation of climbing fibers and PFs (Ito et al., 1982; Sakurai, 1987), and PF stimulation may be replaced with application of NO (Lev-Ram et al., 1995). This LTD is thought to reflect desensitization of postsynaptic Glu receptors (Ito et al., 1982; Linden et al., 1991; Nakazawa et al., 1995). LTP of PF–Purkinje cell synapses is also elicited by Br-cGMP when EGTA is injected into the Purkinje cells (Shibuki and Okada, 1992). Because soluble guanylate cyclase is localized in Purkinje cells (Ariano et al., 1982), this LTP might be of postsynaptic origin. Presynaptic LTD has been demonstrated in the mossy fiber synapses (Kobayashi et al., 1996; Yokoi et al., 1996). Because there is a similarity between presynaptic LTP in mossy fiber synapses and in PF–Purkinje cell synapses, PF–Purkinje cell synapses may also exhibit presynaptic LTD. Therefore, theoretically four types of synaptic plasticity can be present in PFs–Purkinje cell synapses. Plasticity of NO release might serve as a coordinator between presynaptic and postsynaptic plasticity in PF–Purkinje cell synapses. Changes in the ability to induce synaptic plasticity are known in various synapses, and these are referred to metaplasticity (Abraham and Bear, 1996). Dynamic changes in NO release could cause metaplasticity in synapses that exhibit NO-dependent synaptic plasticity.

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