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ACCELERATED COMMUNICATION

Novel Action of Nitric Oxide as Mediator of *N*-Methyl- D-aspartate-Induced Phosphatidylinositol Hydrolysis in Neonatal Rat Cerebellum

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SUMMARY

Nitric oxide (NO) is an intercellular mediator produced within the cerebellum and other central nervous system sites. Results from the present study suggest a novel role for this gaseous second messenger in mediating the stimulatory actions of the excitatory amino acid agonist *N*-methyl-D-aspartate (NMDA) on turnover of phosphatidylinositol (PI) in the neonatal cerebellum. Activation of the NMDA receptor stimulates PI turnover in developing cerebellum when these neurons are in a depolarized state, but the mechanism underlying this effect is unknown. We measured changes in PI hydrolysis induced by NMDA in the presence of baclofen, which is known to depolarize neurons by activating presynaptic inhibitory γ-aminobutyric $acid_B$ autoreceptors. NMDA increased PI hydrolysis by 80% in the presence of 1 μM baclofen. This modulatory action of NMDA was prevented by two competitive inhibitors of NO synthase, L-N^G-monomethylarginine and L-N_ω-nitroarginine, as well as by hemoglobin, which binds NO. Inhibition of NMDA-induced PI hydrolysis by L-N^G-monomethylarginine was reversed by prior administration of L-arginine (200 μM), the physiological substrate of NO synthase. Arginine (500 μM) alone was also able to increase PI hydrolysis significantly. Superoxide dismutase, which prolongs the half-life of NO, also significantly increased the ability of NMDA to stimulate PI hydrolysis. However, NO-induced activation of the cGMP pathway did not appear to be responsible for the NMDA-induced increase in PI hydrolysis, because addition of 8-bromo-cGMP decreased this parameter, and methylene blue, which blocks guanylate cyclase activity, did not inhibit the PI hydrolysis evoked by NMDA receptor activation. These results suggest that NMDA receptor activation acts to release NO, which then acts through a novel pathway to enhance the hydrolysis of PI in the developing rat cerebellum. This novel role for NO in mediating the stimulatory actions of NMDA on PI hydrolysis may be important for developmental processes in the central nervous system.

Ongoing studies from this laboratory have demonstrated that, under depolarizing conditions, NMDA receptor stimulation produces robust increases in PI hydrolysis in the rat cerebellum, assessed at postnatal day 7 (1). One means by which this depolarization can occur is via stimulation of presynaptic $GABA_B$ autoreceptors by low concentrations of baclofen, an action that decreases GABA release (2–4). Disinhibition of the postsynaptic neuron by this mechanism can result in sufficient depolarization to permit NMDA receptor activation (5). Elevations in IP levels produced under these conditions would result in increases in intracellular calcium primarily via abundant cerebellar inositol trisphosphate receptors (6). Calcium plays a role in initiating dendritic potentials in cerebellar Purkinje cells and can exert trophic effects on multiple cell types (7–9). Therefore, alterations in PI hydrolysis with subsequent changes in internal Ca^{2+} levels may have important consequences for cerebellar development.

In the present study, we have tested the hypothesis that the novel second messenger NO mediates these stimulatory effects of NMDA on PI metabolism in the developing cerebellum. NMDA-induced production of NO was first described by Garthwaite *et al.* (10, 11) and Bredt and Snyder (12) in neonatal cerebellum. The NO synthase system has been identified subsequently in granule cells, in basket cells, and in mossy fiber afferents to the cerebellum (13, 14). NO produced by NMDA receptor activation elevates levels of cGMP in the cerebellum (12, 15), as well as in the hippocampus (16). A role for NO in cerebellar and hippocampal plasticity has also been demonstrated (17–19). However, the possibility exists that, as a gas, NO may activate novel second messenger systems because of its widespread sphere of influence.

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[1] S. Smith and J. Li, unpublished observations.

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; IP, inositol phosphate; PI, phosphatidylinositol; SOD, superoxide dismutase; Hb, hemoglobin; MB, methylene blue; GABA, γ-aminobutyric acid; NO, nitric oxide; QUIS, quisqualate; L-NMMA, L-N^G-monomethylarginine.

Materials and Methods

In this study, the role of the NO system in mediating enhanced levels of PI turnover was tested using [^{3}H]inositol (Amersham) in an assay modified from that described by Fisher *et al.* (20). PI hydrolysis was evaluated by assessing IP formation from [^{3}H]PI in 160-μm cross-chopped slices of cerebellar tissue from female rats at postnatal days 7, 14, or 21 or from adult animals. Uptake of [^{3}H]inositol took place over a 90-min period at 37° under an atmosphere of 95% O_2/5% CO_2 in a Ca^{2+}-free buffer. Accumulation of [^{3}H]IPs was quantified after exposure of cerebellar slices to excitatory amino acids for a 20-min period in a 3 mM Ca^{2+} buffer. Excitatory amino acid treatment of tissue was preceded by a 5-min incubation with 1 μM baclofen, 10 mM LiCl, and 500 nM tetrodotoxin. The reaction was terminated by the addition of chloroform/methanol (2:1, v.v) with HCl, and the organic phase was removed. Total IPs in the aqueous phase were separated using anion exchange chromatography (Dowex 1 × 8) with ammonium formate/formic acid in a range of concentrations (8:1 to 2:1) to elute individual IPs (21). The rate of PI hydrolysis was then expressed as a ratio of IPs formed versus PI remaining in the organic phase and was converted to a percentage of basal levels. Tissue was exposed to a variety of amino acid agonists or antagonists (NMDA, 100 μM; QUIS, 1 μM; baclofen, 1–100 μM; 2-OH-saclofen, 200 μM). In some cases inhibitors of the NO synthase system (22–24), i.e., L-NMMA (100 μM) or nitro-arginine (100 μM), were added to the incubation solution to test the possible involvement of NO in mediating the potentiating effect of NMDA on PI hydrolysis observed in the presence of 1 μM baclofen. Other agents tested include arginine (30 μM to 10 mM), Hb (5 μM), and SOD (30 units/ml). Drugs were added to the incubating cerebellar slices 5 min before addition of baclofen and/or NMDA. In order to prevent oxidation of Hb before NO chelation, 30 mM degassed $FeSO_4$ was mixed with Hb in a container coated with 1% bovine serum albumin. Before administration of this agent, the Hb solution was exposed to high pressure N_2 gas to replace atmospheric oxygen.

In order to test the role of the cGMP system in mediating excitatory amino acid-induced elevations in PI hydrolysis, two additional experiments were performed; MB (10 μM) or 8-bromo-cGMP (1 mM) was added 5 min before addition of baclofen and NMDA to the incubation medium, in separate experiments, and the PI turnover rate was determined. All compounds were obtained from Research Biochemicals Inc. (Natick, MA) or from Sigma Chemical Co. (St. Louis, MO).

Results and Discussion

Baclofen increased NMDA-stimulated values of PI hydrolysis in a concentration-dependent fashion (Fig. 1A). Maximal levels of PI hydrolysis (an 80% increase above basal values, $p < 0.001$) were observed in the presence of a 1 μM concentration of baclofen (Fig. 1A). At this concentration, baclofen activates presynaptic $GABA_B$ autoreceptors (2–4). Higher concentrations of baclofen (200 μM) depressed NMDA-induced levels of PI hydrolysis (1). Production of inositol monophosphate and of inositol trisphosphate were enhanced by equivalent amounts under the former experimental conditions (data not shown). In contrast, neither basal nor QUIS-stimulated levels of PI hydrolysis were altered by baclofen applied in concentrations of 500 nM to 200 μM. Enhancement of NMDA-stimulated levels of PI turnover was due to stimulation of the $GABA_B$ receptor by baclofen, because 200 μM 2 OH-saclofen blocked this effect (Fig. 1A). Furthermore, the permissive effect of baclofen on NMDA-stimulated elevations in PI hydrolysis was mimicked by combined administration of both bicuculline (50 μM) and 2 OH-saclofen (50 μM), at doses that block postsynaptic $GABA_A$ and $GABA_B$ receptors, respectively (1). This finding also suggests that depolarizing conditions resulting from reduced GABA inhibition permit NMDA-induced increases in PI hydrolysis. Similar results have been obtained with K^+ or under Mg^{2+}-free conditions (25), which also permit elevated levels of NMDA-induced PI hydrolysis.

The permissive effect of baclofen on the ability of NMDA to stimulate levels of PI hydrolysis above basal values varies over

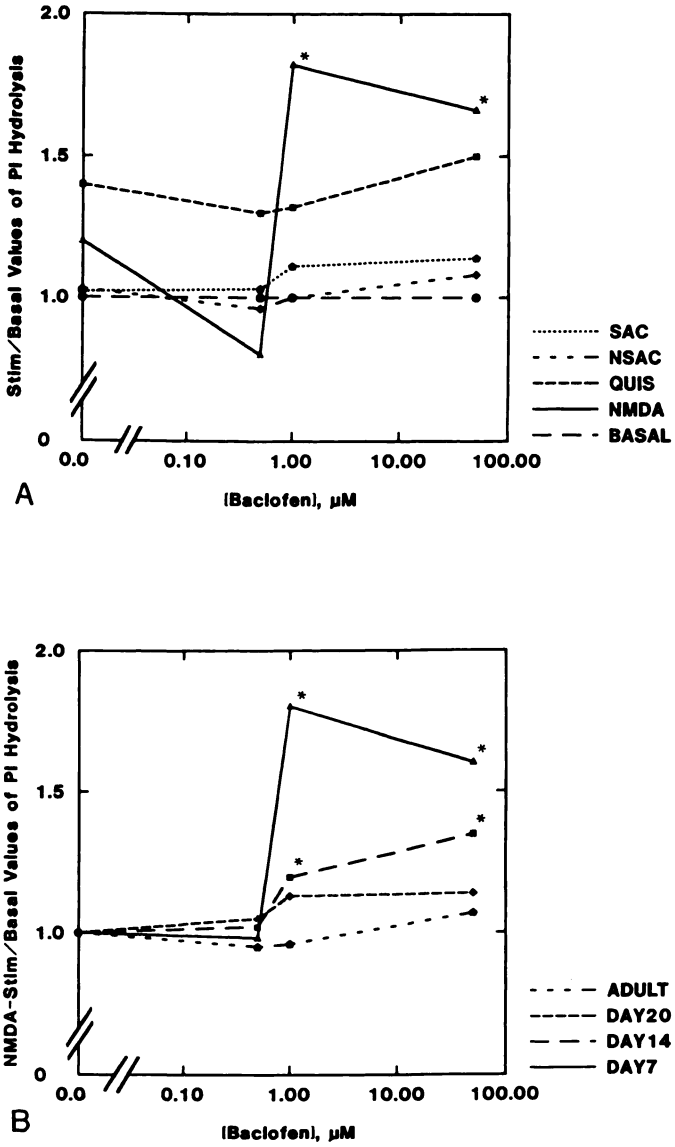

Fig. 1. $GABA_B$ receptor stimulation by baclofen enhances levels of PI hydrolysis stimulated by NMDA in neonatal cerebellum. The PI turnover rate was expressed as a ratio of IPs formed versus PI remaining in the organic phase. Data are means ± standard errors of 10–16 determinations from five to eight experiments. A, A concentration-dependent permissive effect of baclofen in facilitating PI hydrolysis induced by NMDA, but not by QUIS, above control levels is observed. Concentrations of baclofen from 1 to 50 μM were effective, with the 1 μM dose being the most significant. The facilitative effect of baclofen on NMDA-stimulated levels of PI hydrolysis was blocked by prior administration of the specific $GABA_B$ antagonist 2 OH-saclofen (200 μM), suggesting that activation of $GABA_B$ receptors is involved in this interaction. Baclofen alone, however, did not significantly alter levels of PI hydrolysis. Drug groups were basal (*BASAL*), NMDA (100 μM), QUIS (1 μM), 2 OH-saclofen (*SAC*) (200 μM), and NMDA plus 2 OH-saclofen (*NSAC*). B, The permissive effect of 1 μM baclofen on NMDA-stimulated levels of PI hydrolysis in cerebellar slices is observed maximally at postnatal day 7 and declines with age. Insignificant levels of NMDA-stimulated PI turnover are noted by day 20 and in the adult. *, $p < 0.05$ versus basal values obtained in the absence of added baclofen or NMDA.

postnatal development. NMDA-stimulated levels of PI hydrolysis reached maximal levels at postnatal day 7 (Fig. 1B) and subsequently declined, reaching basal levels by postnatal day 20. Postnatal day 7 is especially significant for development because climbing fiber and mossy fiber innervation of Purkinje and granule cells, respectively, is culminating at this time. Maximal levels of PI hydrolysis evoked by stimulation of metabotropic receptors are also seen at this age (26).

The NMDA-induced increase in PI hydrolysis observed in the neonatal cerebellum appears to be mediated by the second messenger NO. The stimulatory effect of NMDA on PI hydrolysis was completely prevented by 100 μM concentrations of either L-NMMA or nitro-arginine (22–24) (Figs. 2 and 3). The L-NMMA blockade could be reversed by 200 μM arginine (Fig. 2). In addition, a 30 μM concentration of arginine could substitute for either baclofen or NMDA in stimulating PI turnover 50% above basal values (Table 1). When administered at a 500 μM concentration, arginine alone was able to stimulate PI turnover levels 30% above basal values, suggesting a direct action of NO on the PI system. A higher concentration of arginine (10 mM) produced levels of PI turnover equivalent to maximal levels observed in the presence of 100 μM NMDA and 1 μM baclofen (Table 1). However, in no case did addition of arginine at any concentration produce levels of PI turnover greater than those observed in the presence of NMDA and baclofen, suggesting that this maximal value is independent of substrate availability.

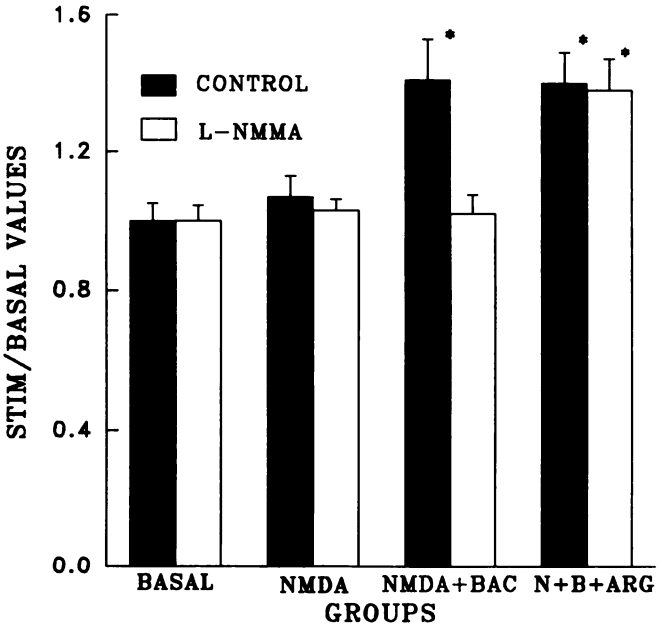

Fig. 2. NMDA-stimulated levels of PI turnover observed under low levels of $GABA_B$ receptor stimulation are blocked by the NO synthase inhibitor L-NMMA (100 μM), an effect reversed by the addition of a 200 μM concentration of L-arginine (*ARG*), the substrate for this enzyme. Levels of PI hydrolysis are expressed as a ratio of stimulated/control, basal values. The arginine compounds were added to the incubation buffer 5 min before addition of the amino acid analogs. L-NMMA alone produced no effect on PI hydrolysis under basal, NMDA-stimulated or baclofen-stimulated conditions (the latter data are not indicated). Data are means ± standard errors of 8–10 determinations from four or five experiments. Average aqueous cpm for significant drug groups were as follows: basal, 950; NMDA, 975; NMDA plus baclofen groups, control, 1475; L-NMMA, 980; arginine 1450; arginine plus L-NMMA, 1430. Counts from the organic phase varied from 10,000 to 20,000 cpm across different experiments. Groups were basal, NMDA (100 μM), NMDA plus baclofen (*BAC*) (1 μM), and NMDA plus baclofen plus L-arginine (200 μM) (*N+B+ARG*). *, $p < 0.001$ versus control basal values.

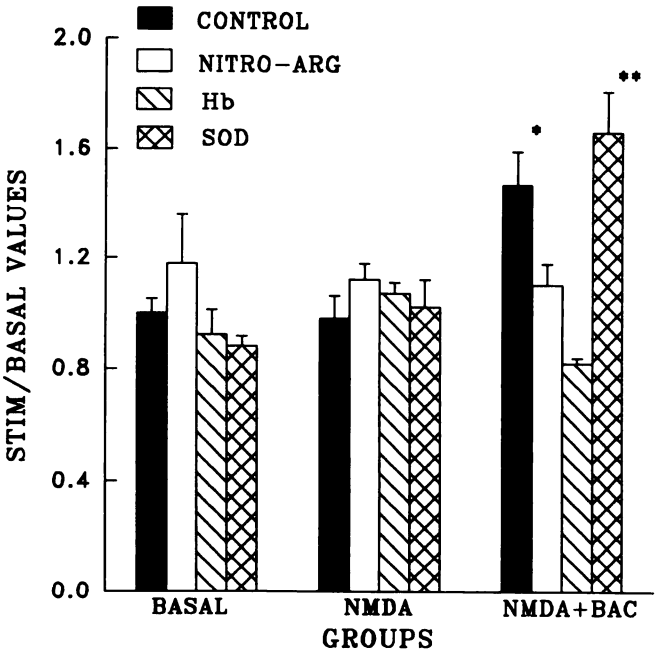

Fig. 3. Alterations in NO or NO synthase activity influence the magnitude of NMDA-stimulated levels of PI turnover observed under conditions of low level $GABA_B$ receptor stimulation [NMDA, 100 μM; baclofen (*BAC*), 1 μM]. Agonist-stimulated values are expressed relative to control, basal values. Drugs tested were *L-Nω*-nitroarginine (*NITRO-ARG*) (100 μM) (NO synthase inhibitor), Hb (5 μM) (NO chelator), and SOD (30 units/ml), which prevents oxidation and thus prolongs the half-life of NO. Drugs were added to the incubating cerebellar slices 5 min before addition of baclofen and/or NMDA. Both nitro-arginine and Hb administration completely blocked the permissive effect of 1 μM baclofen on NMDA-stimulated levels of PI turnover. In contrast, addition of SOD potentiated the synergistic effect of baclofen/NMDA administration on PI hydrolysis ($p < 0.05$). In no case did any drug treatment alter levels of PI hydrolysis observed in the presence of baclofen (1 μM). Data are means ± standard errors of six to eight determinations from three or four experiments. Average aqueous cpm from significant drug groups were as follows: basal, 700; NMDA, 680; NMDA plus baclofen groups, control, 1250; Hb, 605; SOD, 1400; nitro-arginine, 808. Counts from the organic phase varied from 10,000 to 20,000 cpm across experiments. *, $p < 0.001$ versus control basal values. **, $p < 0.05$ versus levels obtained in the presence of baclofen and NMDA.

Alterations in the availability of NO produced significant changes in the ability of NMDA to stimulate PI hydrolysis above basal values. The half-life of NO, which is normally 1–6 sec *in vitro*, can be enhanced 2-fold with the addition of 30 units/ml SOD to the incubation medium (10). Under such conditions, prolonged activity of this second messenger produced a 10% increase in levels of PI turnover stimulated by NMDA and baclofen (Fig. 3). Another agent, Hb, which binds NO, was tested for its effect on NMDA stimulation of PI turnover. Administration of 5 μM Hb using a gas delivery system, as described previously (10), depressed NMDA/baclofen-stimulated values of PI turnover by 45% (Fig. 3), thus also supporting the view that NO production is essential for NMDA-induced stimulation of PI hydrolysis.

NO is known to stimulate cGMP formation in neonatal and adult cerebellum (12, 15, 16). However, cGMP can either enhance or depress IP formation, depending on the site studied (27, 28). Therefore, the role of cGMP in mediating NMDA-induced PI hydrolysis was tested in this system using MB, a

TABLE 1

L-Arginine, the substrate of NO synthase, can substitute for either NMDA or baclofen in potentiating PI hydrolysis in neonatal cerebellar tissue

Using procedures described previously, either L-arginine (30 μM, 500 μM, or 10 mM), baclofen (1 μM), L-arginine plus baclofen, or L-arginine plus L-NMMA (100 μM) were added to the incubation buffer 5 min before NMDA (100 μM). Levels of PI hydrolysis were then determined as a percentage of control basal values. L-Arginine (30 μM) in combination with NMDA or baclofen produced levels of PI hydrolysis (47–57% above basal) similar to those observed after exposure of cerebellar slices to baclofen and NMDA. This permissive effect of L-arginine could be blocked with prior administration of L-NMMA, an inhibitor of NO synthase. In addition, at the two higher concentrations (500 μM and 10 mM), L-arginine alone produced a significant ($p < 0.05$) elevation in levels of PI hydrolysis above basal values. These results suggest that enhanced production of NO facilitates PI hydrolysis. These results are means ± standard errors of 8–10 determinations from four or five experiments. Average aqueous cpm from significant drug groups were as follows: basal, 850; NMDA, 863; NMDA plus baclofen, 1350; NMDA plus arginine (30 μM), 1325; arginine (500 μM), 1105; arginine (500 μM) plus NMDA, 1380; arginine (10 mM), 1352; arginine (10 mM) plus NMDA, 1312; arginine (30 μM) plus baclofen, 1250; arginine (30 μM) plus baclofen plus NMDA, 1385. Counts from the organic phase varied from 10,000 to 20,000 cpm across experiments.

	PI hydrolysis	
	Basal	NMDA
	% of control basal values	
Control	100	104 ± 6.4
Baclofen	101 ± 2.1	157 ± 6.2*
Arginine (30 μM)	107 ± 3.7	154 ± 5.4*
Arginine (500 μM)	130 ± 3.1*	159 ± 5.7*
Arginine (10 mM)	157 ± 7.5*	152 ± 6.2*
Arginine (30 μM) + L-NMMA	116 ± 3.2	84 ± 1.5
Arginine (30 μM) + baclofen	147 ± 5.6*	163 ± 4.3*

*$p < 0.05$ versus control basal values.

drug that blocks NO-induced stimulation of the guanylate cyclase system (10, 29). At a 10 μM concentration, MB did not prevent elevations in PI hydrolysis produced by NMDA and baclofen (Fig. 4). In addition, 1 mM 8-bromo-cGMP failed to stimulate levels of PI hydrolysis above basal values and, in fact, reduced to control values NMDA-stimulated elevations in PI hydrolysis observed in the presence of low concentrations of baclofen (Fig. 4). It is also noteworthy that the NMDA-NO system stimulates cGMP formation under conditions different from those required for stimulation of PI hydrolysis in the present system (i.e., the former event does not require 1 μM baclofen). In sum, the results of these experiments strongly suggest that stimulation of the cGMP system by NO does not mediate the potentiating effect of this second messenger on PI metabolism.

Recent studies have localized IP formation in the cerebellum to Purkinje cells (30) and granule cells (25). Granule cells are the most likely cerebellar site for NMDA/baclofen stimulation of NO with subsequent activation of PI hydrolysis. Granule cells contain both NO synthase (14) and NMDA receptors (31, 32). These receptors would be activated by glutamate released from mossy fiber afferents during movement. Maximal levels of NMDA receptor activation would be observed when granule cells are sufficiently depolarized, as would occur when they are disinhibited via stimulation of presynaptic $GABA_B$ autoreceptors on synapsing Golgi cells. Under these conditions, NMDA receptor activation would release NO and produce increases in PI hydrolysis. Byproducts of PI hydrolysis, i.e., IPs and diacylglycerol, are agents that can then act to elevate intracellular calcium and phosphorylate cellular proteins, respectively, events that would facilitate neurogenesis (33).

NO may also act as a retrograde messenger to alter presynaptic release of transmitters within a local network, as has been

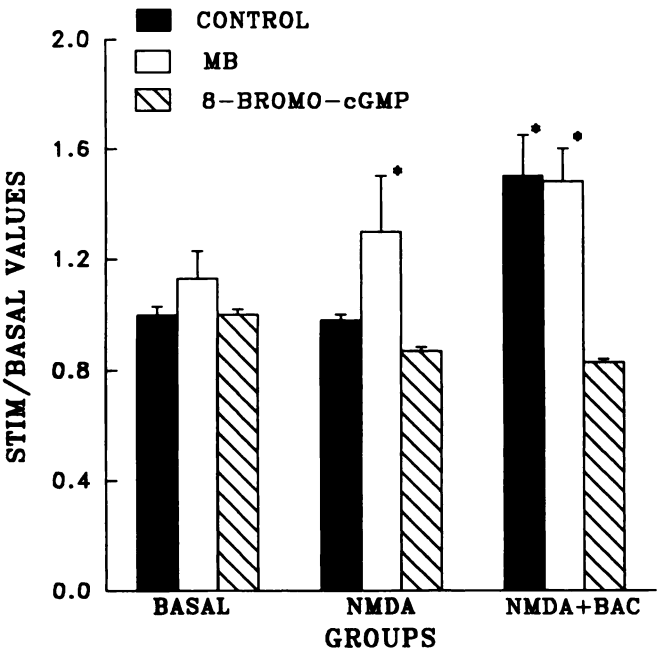

Fig. 4. NMDA-stimulated levels of PI hydrolysis via NO during $GABA_B$ receptor activation are not dependent upon cGMP formation. Agonist-stimulated values of PI turnover are presented as a ratio of stimulated/control basal values (*BASAL*). MB (10 μM), which prevents cGMP formation, or 8-bromo-cGMP (1 mM) was added 5 min before addition of baclofen (1 μM) and NMDA (100 μM) (*NMDA + BAC*) or NMDA alone (*NMDA*) to incubating cerebellar slices. Addition of MB produced no effect on NMDA-stimulated levels of PI turnover in the presence of baclofen, although MB alone significantly increased NMDA-induced PI turnover (without baclofen). In contrast, 8-bromo-cGMP significantly decreased NMDA/baclofen-stimulated levels of PI turnover. This compound alone had no effect on either basal levels or NMDA-stimulated levels. Neither drug altered levels of PI hydrolysis observed in the presence of baclofen alone. Results are mean ± standard error of 8–10 determinations from four or five experiments. Average aqueous cpm from significant drug groups were as follows: basal, 780; NMDA, 775; NMDA plus MB, 1014; NMDA plus baclofen groups, control, 1305; MB, 1293; 8-bromo-cGMP, 650. Counts from the organic phase varied from 10,000 to 20,000 cpm across experiments. *, $p < 0.01$ versus control basal values.

described (34). A presynaptic action of NO in releasing norepinephrine is not likely to mediate the stimulation of PI hydrolysis observed in the present study, however, because administration of the α_1-adrenoreceptor antagonist prazosin (1 μM) did not block NMDA-induced stimulation of PI hydrolysis.[1]

The data from the present study demonstrate a novel action of the NO system in stimulating PI hydrolysis in the neonatal cerebellum, distinct from its actions on guanylate cyclase. In addition, both second messenger systems, IPs and NO, have been suggested as possible cellular mediators (17, 18, 33, 35) of long term depression in the cerebellum (36). Results from this study provide a potential link between these signal transducers, which may also be important for plasticity in the adult cerebellum, as well as during development.

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