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Inhibitory and Potentiating Influences of Glycine on *N*-Methyl-D-aspartate-Evoked Dopamine Release from Cultured Rat Mesencephalic Cells

HOWARD MOUNT, REMI QUIRION, ISABELLE CHAUDIEU, and PATRICIA BOKSA

Douglas Hospital Research Center, Departments of Psychiatry and of Pharmacology and Therapeutics, McGill University, Verdun, Quebec, Canada H4H 1R3

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SUMMARY

In the presence of 1.2 mM Mg^{2+}, glycine (30–100 μM) inhibited [^{3}H]dopamine ([^{3}H]DA) release stimulated by *N*-methyl-D-aspartate (NMDA), in fetal rat mesencephalic cell cultures. Strychnine (1 μM) blocked the inhibitory effect of 100 μM glycine, indicating an action via strychnine-sensitive inhibitory glycine receptors. A higher concentration of strychnine (100 μM), by itself, inhibited NMDA-evoked [^{3}H]DA release in the presence or absence of Mg^{2+}. Spontaneous [^{3}H]DA release and [^{3}H]DA release stimulated by kainate and quisqualate were unaffected by glycine (≤100 μM) or strychnine (≤100 μM), indicating that glycine and strychnine modulatory effects are only associated with the NMDA receptor subtype. [^{3}H]DA release evoked by K^+ (56 mM) was unaffected by glycine (≤100 μM) but was attenuated by a high concentration of strychnine (100 μM). In the absence of exogenous Mg^{2+}, glycine (30–100 μM) potentiated NMDA-evoked [^{3}H]DA release by a strychnine-insensitive mechanism. A selective antagonist of the NMDA-associated glycine receptor, 7-chlorokynurenate (10 μM), attenuated NMDA-evoked [^{3}H]DA release in the absence of Mg^{2+}. The effect of 10 μM 7-chlorokynurenate was overcome by 1 μM glycine. Also, when tested in the presence of 1.2 mM Mg^{2+} and 1 μM strychnine, 100 μM 7-chlorokynurenate inhibited NMDA-evoked [^{3}H]DA release, and this antagonism was overcome by 30 to 100 μM glycine. These results indicate that two distinct glycine receptors modulate NMDA-stimulated [^{3}H]DA release from mesencephalic cells in culture. Manipulation of extracellular Mg^{2+} permits the differentiation of a strychnine-sensitive glycine response (inhibition of NMDA-evoked [^{3}H]DA release) from a strychnine-insensitive glycine response (potentiation of NMDA-evoked [^{3}H]DA release). It is suggested that voltage-dependent Mg^{2+} blockade of the NMDA response may allow for the expression of these opposing effects of glycine.

The NMDA receptor is under the control of various modulators, including the divalent cation Mg^{2+} and the amino acid glycine. Activity at the receptor is subject to voltage-dependent inhibition by low millimolar concentrations of Mg^{2+} (1–4). Thus, the blockade of NMDA responses may be alleviated in partially depolarized neurons (5). Glycine acts at a strychnine-insensitive site that is allosterically associated with the NMDA receptor to potentiate NMDA receptor-mediated responses (Ref. 6; also reviewed in Ref. 7).

NMDA receptors have been found to mediate release of DA or [^{3}H]DA from rat brain slices of substantia nigra (8, 9), striatum (8, 10–14), and nucleus accumbens (8, 15), from cultured cells of fetal rat ventral mesencephalon (16), and in trans-striatal dialysis experiments (17). In striatal slices, NMDA receptor activation resulted in DA release that was consistently inhibited by Mg^{2+} (8, 10–13). However, exogenous glycine failed to potentiate NMDA-evoked release of [^{3}H]DA (18) or endogenous DA (14). Despite this observation, kynurenate, an agent that blocks NMDA responses through competitive inhibition of glycine binding at the strychnine-insensitive glycine site (19–21), did antagonize NMDA-stimulated [^{3}H]DA release from the slices (18). It is, therefore, plausible that, in the absence of exogenous glycine and kynurenate, a potentiating effect of glycine on NMDA-stimulated [^{3}H]DA release is produced by endogenous glycine, present in the striatal slice at levels sufficient to maximally saturate this glycine binding site.

Dopaminergic transmission may also be modulated by the actions of glycine at an inhibitory (hyperpolarizing) glycine receptor, which is selectively blocked by 1 to 10 μM strychnine (22, 23). Strychnine-sensitive glycine receptors have been demonstrated in brain regions enriched in dopaminergic cell bodies and nerve terminals (24–27) and have been implicated in in-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; DA, dopamine; EAA, excitatory amino acid; KRH, Krebs-Ringer-HEPES buffer; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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hibition of DA neuron firing in the substantia nigra zona compacta (28, 29) and modulation of DA release from striatum (Refs. 10, 30, and 31, but see Ref. 14), substantia nigra (9, 32), and ventral tegmentum (33).

We previously reported (16) that NMDA, quisqualate, and kainate each evoked Ca^{2+}-dependent release of [^{3}H]DA from dissociated cell cultures of fetal rat mesencephalon. The pattern of antagonist selectivity and ionic sensitivity of release stimulated by NMDA, quisqualate, and kainate suggested that the responses were mediated by distinct NMDA and non-NMDA EAA receptors.

In this study, we tested effects of glycine on spontaneous and EAA-evoked [^{3}H]DA release from mesencephalic cell cultures. We show that either inhibitory or potentiating effects of glycine on NMDA-evoked [^{3}H]DA release may be demonstrated through manipulation of extracellular Mg^{2+} concentrations. We suggest that it is the unique property of the NMDA receptor to exhibit voltage-dependent Mg^{2+} blockade that allows glycine to produce these opposing effects on the NMDA response.

Experimental Procedures

Materials. 3,4-[8-^{3}H]Dihydroxyphenylethylamine ([^{3}H]DA, 20 Ci/mmol) was purchased from New England Nuclear (Boston, MA). NMDA, quisqualate, kainate, desipramine hydrochloride, and pargyline hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Glycine, strychnine phosphate, and 7-chlorokynurenate were from Fisher Scientific (Fair Lawn, NJ), the New York Quinine Co. (New York), and Research Biochemicals Inc. (Natick, MA), respectively. Other chemicals were reagent grade from regular commercial sources.

Methods. Dissociated cell cultures were prepared from anterior ventral mesencephalic tissue of Sprague-Dawley rat fetuses (gestation day 15), as previously described (34). Cell cultures were grown in 24-well multiwell plates (fetal tissue from a single dam was divided between 24 to 36 culture wells) in 0.5 ml of Dulbecco's modified Eagle's medium, supplemented with 10% (v/v) fetal calf serum and 20 mM KCl.

After 6–7 days in culture, cells were rinsed with KRH [pH 7.4, composed of (in mM): NaCl, 125; KCl, 4.8; HEPES, 25; NaOH, 5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; D-glucose, 5.6; $CaCl_2$, 2.2; pargyline, 0.1; ascorbate, 1.0] and loaded with [^{3}H]DA (50 nM, 20-min incubation), in the presence of desipramine (50 μM). After [^{3}H]DA loading, each well was rinsed four times with KRH. KRH from a 5-min incubation (spontaneous release period), which was followed immediately by a 5-min incubation in the presence of an EAA agonist (stimulated release period), was collected, and [^{3}H]DA release was quantitated by measurement of radioactivity in each aliquot of buffer collected. In earlier work, more than 95% of the radioactivity detected in buffer collected after spontaneous and glutamate-stimulated release periods was determined by high performance liquid chromatography to be [^{3}H]DA (34). Also, more than 90% of the total cell content of tritiated species, assayed immediately before release collection periods, was found to be [^{3}H]DA. Following release incubations, radioactivity remaining in the cells was extracted by a 30-min incubation with acidified ethanol (95% ethanol/5% 0.1 M HCl). When antagonists were tested, cells were exposed to these during the spontaneous release incubation as well as during exposure to the agonist. In experiments performed in the absence of Mg^{2+}, $MgSO_4$ was omitted from the KRH.

For data presentation, spontaneous [^{3}H]DA release (typically 3–5% of total intracellular [^{3}H]DA stores) was subtracted from release stimulated by the EAA agonist. The net release evoked by the agonist was expressed as a percentage of the total [^{3}H]DA uptake into cells. Reported values are means of at least six determinations (six cell culture wells from fetal tissue of four or more pregnant dams). Statistically significant differences between means were determined by Student's *t* test for unpaired observations or by one-way analysis of variance and *post hoc* Newman-Keul's test for multiple comparisons, as appropriate. The accepted level of statistical significance was $\alpha = 0.05$.

Results

Effects of glycine on EAA- and K^+-evoked [^{3}H]DA release in the presence of 1.2 mM Mg^{2+}. As we have previously reported (16, 34), NMDA (100 μM) (Fig. 1), quisqualate (10 μM), kainate (100 μM), and K^+ (56 mM) (Table 1) stimulated [^{3}H]DA release from mesencephalic cell cultures. In the presence of 1.2 mM Mg^{2+}, exogenous glycine (30–100 μM) selectively blocked the NMDA response (Fig. 1), without affecting spontaneous [^{3}H]DA release or [^{3}H]DA release stimulated by quisqualate, kainate, or K^+ (Table 1). Strychnine (0.1 to 1 μM) induced a concentration-dependent blockade of this inhibitory effect of glycine (Fig. 2A).

Control experiments showed that, in the absence of exogenous glycine, the NMDA response was unaffected by 1 or 10 μM strychnine but was blocked by 100 μM strychnine (Table 2). This confirms that 1 μM is an appropriate concen-

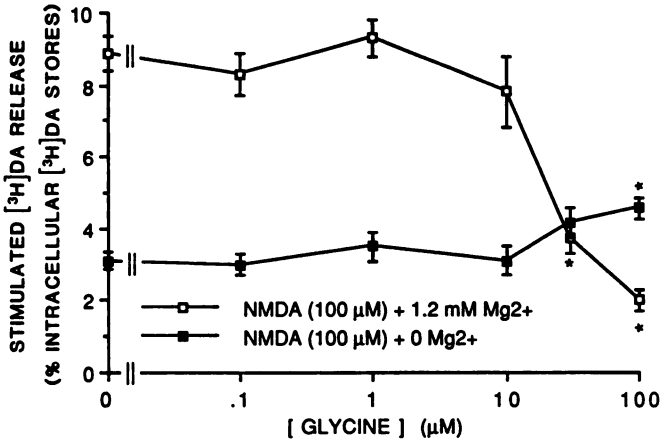

Fig. 1. Effect of glycine on [^{3}H]DA release evoked by NMDA, in the presence of 1.2 mM Mg^{2+} and under nominally Mg^{2+}-free conditions. Cells were exposed to glycine (0.1–100 μM) during the spontaneous incubation and in the presence of NMDA (100 μM). Spontaneous [^{3}H]DA release has been subtracted from [^{3}H]DA release stimulated by NMDA. Results are the means ± standard errors from six to 12 cultures. *, $p < 0.05$, compared with control NMDA-evoked [^{3}H]DA release.

TABLE 1

Effect of glycine on spontaneous [^{3}H]DA release and on [^{3}H]DA release evoked by quisqualate, kainate, and K^+

Spontaneous [^{3}H]DA release and release in the presence of glycine, quisqualate, kainate, or K^+ were collected in standard KRH or in KRH containing 100 μM glycine. Spontaneous [^{3}H]DA release has been subtracted from release evoked by the test compound. The resultant stimulated release is expressed as a percentage of total [^{3}H]DA uptake into cells. Each value is the mean ± standard error from six to 11 cultures.

Test compounds	Stimulated [^{3}H]DA release
	% of intracellular [^{3}H]DA stores
Glycine (100 μM)	−0.15 ± 0.3[a]
Quisqualate (10 μM)	4.3 ± 0.2
Quisqualate (10 μM) + glycine (100 μM)	3.8 ± 0.3[b]
Kainate (100 μM)	11.7 ± 0.9
Kainate (100 μM) + glycine (100 μM)	11.7 ± 1.0[b]
K^+ (56 mM)	26.8 ± 0.9
K^+ (56 mM) + glycine (100 μM)	28.8 ± 1.6[b]

[a] Not different from spontaneous [^{3}H]DA release.
[b] Not different from [^{3}H]DA release stimulated by the test compound in the absence of glycine.

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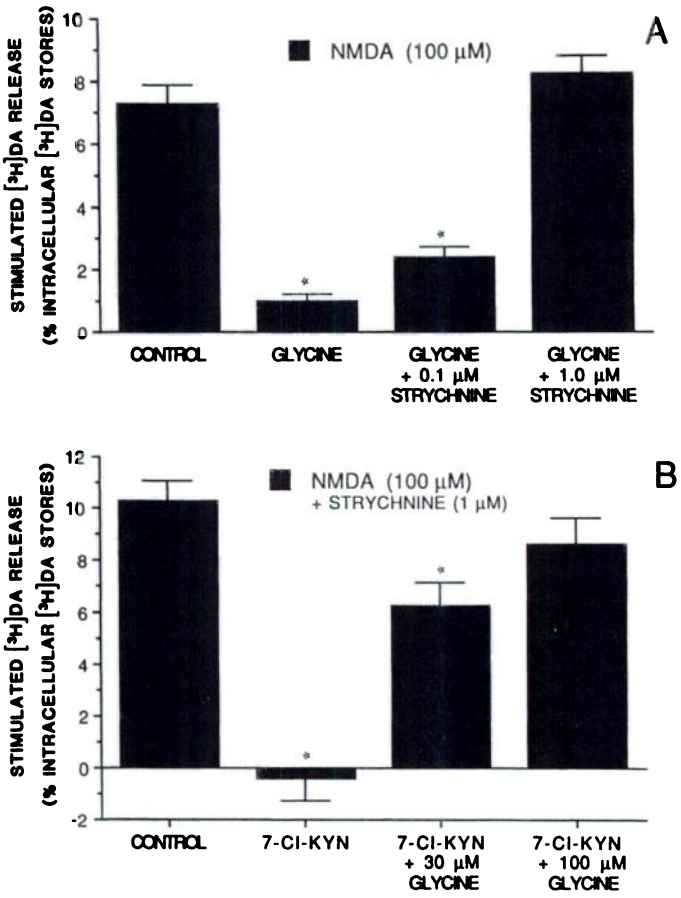

Fig. 2. Effect of glycine, with or without strychnine, on [^{3}H]DA release evoked by NMDA (A), and effect of 7-chlorokynurenate (*7-Cl-KYN*) (with strychnine) on the potentiation by endogenous glycine of the NMDA response (B), in the presence of 1.2 mM Mg^{2+}. To assess the inhibitory effect of glycine on NMDA-evoked [^{3}H]DA release, cells were exposed to glycine (100 μM), with or without strychnine (0.1 or 1 μM), during the spontaneous incubation and in the presence of NMDA (100 μM). To assess competitive inhibitory effects of 7-chlorokynurenate on the potentiation of NMDA-evoked [^{3}H]DA release by endogenous glycine, cells were exposed to strychnine (1 μM) and 7-chlorokynurenate (100 μM), with or without glycine (30 or 100 μM), during the spontaneous incubation and in the presence of NMDA (100 μM). Spontaneous [^{3}H]DA release has been subtracted from [^{3}H]DA release stimulated by NMDA. Results are the means ± standard errors from nine to 21 cultures. *, $p < 0.05$, compared with control NMDA-evoked [^{3}H]DA release.

tration of strychnine to test the strychnine sensitivity of glycine's inhibitory effect on the NMDA response, as in Fig. 2A. Quisqualate- and kainate-evoked [^{3}H]DA release were unaffected by strychnine [1 μM (data not shown) to 100 μM (Table 2)]. K^+-induced [^{3}H]DA release was unaffected by 1 μM strychnine (data not shown) but was partially attenuated by 100 μM strychnine (Table 2).

Release stimulated by NMDA in the presence of 1.2 mM Mg^{2+} was antagonized by 100 μM 7-chlorokynurenate, a selective antagonist of the glycine binding site associated with the NMDA receptor (35, 36) (Fig. 2B). Exogenous glycine reversed the inhibition of NMDA-evoked [^{3}H]DA release by 7-chlorokynurenate, when the effect of 30 to 100 μM glycine was assessed in the presence of 1 μM strychnine (Fig. 2B).

Effect of glycine on NMDA-evoked [^{3}H]DA release in the absence of Mg^{2+}. When tested in the absence of Mg^{2+}, rather than being inhibited by glycine, the NMDA response was potentiated by 30–100 μM glycine (Fig. 1). [As we have

TABLE 2

Effect of strychnine on spontaneous [^{3}H]DA release and [^{3}H]DA release evoked by NMDA, quisqualate, kainate, and K^+

Spontaneous [^{3}H]DA release and release in the presence of strychnine (1 or 100 μM), NMDA, quisqualate, kainate, or K^+ were collected in standard KRH or in KRH containing the indicated concentration of strychnine. Spontaneous [^{3}H]DA release has been subtracted from release evoked by the test compound. The resultant stimulated release is expressed as a percentage of total [^{3}H]DA uptake into cells. Each value is the mean ± standard error from six to 12 cultures.

Test compounds	Stimulated [^{3}H] DA release
	% of intracellular [^{3}H]DA stores
Strychnine (1 μM)	−0.3 ± 0.4[a]
Strychnine (100 μM)	1.0 ± 0.5
NMDA (100 μM)	7.5 ± 0.8
NMDA (100 μM + strychnine (1 μM)	8.0 ± 1.3
NMDA (100 μM) + strychnine (10 μM)	5.8 ± 0.9
NMDA (100 μM + strychnine (100 μM)	0.8 ± 0.2[ab]
Quisqualate (10 μM)	4.3 ± 0.2
Quisqualate (10 μM) + strychnine (100 μM)	4.3 ± 0.2
Kainate (100 μM)	11.7 ± 0.9
Kainate (100 μM + strychnine (100 μM)	10.6 ± 0.7
K^+ (56 mM)	26.4 ± 0.7
K^+ (56 mM) + strychnine (100 μM)	18.9 ± 0.9[b]

[a] Not different from spontaneous [^{3}H]DA release.
[b] $p < 0.05$ versus [^{3}H]DA release stimulated by the test compound in the absence of strychnine.

previously found (16), removal of Mg^{2+}, by itself, reduced the control NMDA response (compare control NMDA-stimulated [^{3}H]DA release in the presence and absence of added Mg^{2+} in Fig. 1).] Strychnine (1 μM) failed to alter the potentiating effect of 100 μM glycine on NMDA-stimulated [^{3}H]DA release in the absence of Mg^{2+} (Fig. 3A). As observed in the presence of Mg^{2+}, a higher concentration of strychnine (100 μM), when tested by itself, attenuated NMDA-evoked [^{3}H]DA release also in the absence of Mg^{2+} (Fig. 3A).

In the absence of both exogenous Mg^{2+} and strychnine, 10 μM 7-chlorokynurenate attenuated the [^{3}H]DA release evoked by NMDA (Fig. 3B). This inhibition was overcome by 1 μM glycine.

Discussion

Results from this study indicate that two distinct glycine receptors may modulate NMDA-stimulated [^{3}H]DA release from fetal rat mesencephalic cells in culture. Manipulation of extracellular Mg^{2+} permitted the differentiation of a strychnine-sensitive glycine response (inhibition of NMDA-evoked [^{3}H]DA release) from a strychnine-insensitive glycine response (potentiation of NMDA-evoked [^{3}H]DA release). By itself, glycine did not affect spontaneous [^{3}H]DA release.

In the presence of Mg^{2+}, the antagonism of NMDA-stimulated [^{3}H]DA release by 100 μM glycine was probably mediated by the classical inhibitory glycine receptor, because the effect of glycine was blocked in a concentration-dependent manner by low concentrations of strychnine (0.1 or 1 μM). The inhibitory effect of 100 μM glycine was selective for the release evoked by NMDA. It was not observed for quisqualate-, kainate-, or K^+-stimulated [^{3}H]DA release. This observation suggests that the NMDA response may be selectively sensitive to hyperpolarizing effects of inhibitory glycine receptor activation on membrane potential. A less likely possibility is that glycine might interact with the NMDA receptor itself to produce the observed inhibition.

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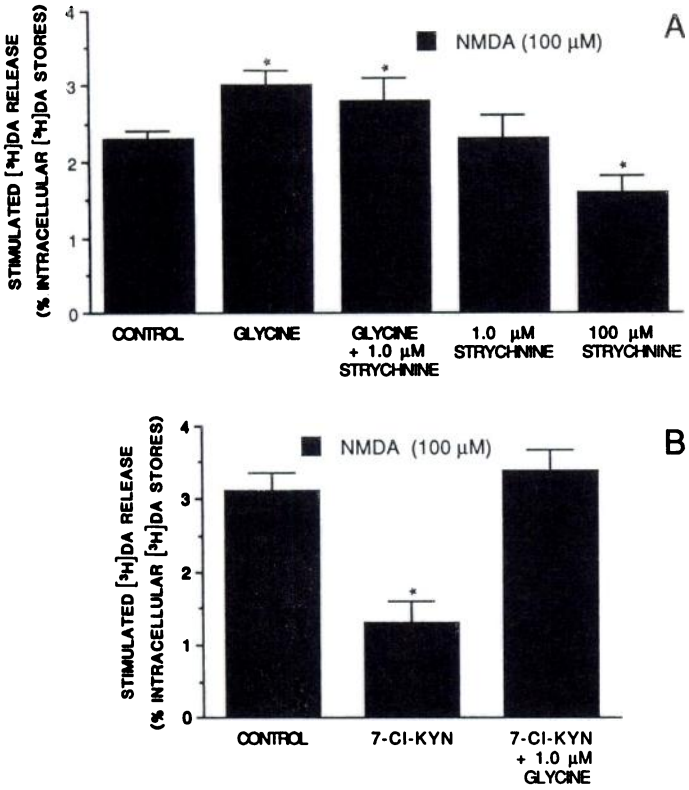

Fig. 3. Effect of glycine, with or without strychnine, on [^{3}H]DA release evoked by NMDA (A), and effect of 7-chlorokynurenate (*7-Cl-KYN*) on the potentiation by endogenous glycine of the NMDA response (B), under nominally Mg^{2+}-free conditions. To assess the potentiating effect of glycine on NMDA-evoked [^{3}H]DA release, cells were exposed to glycine (100 μM), with or without strychnine (1 or 100 μM), during the spontaneous incubation and in the presence of NMDA (100 μM). To assess competitive effects of 7-chlorokynurenate on the potentiation of NMDA-evoked [^{3}H]DA release by exogenous glycine, cells were exposed to 7-chlorokynurenate (10 μM), with or without glycine (1 μM), during the spontaneous incubation and in the presence of NMDA (100 μM). Spontaneous [^{3}H]DA release has been subtracted from [^{3}H]DA release stimulated by NMDA. Results are the means ± standard errors from nine to 12 cultures. *, $p < 0.05$, compared with control NMDA-evoked [^{3}H]DA release.

To determine which of these mechanisms might explain the selectivity for NMDA responses, effects of glycine were compared in the presence and absence of Mg^{2+}. Extracellular Mg^{2+} was manipulated because activity at the NMDA receptor is characteristically inhibited by low millimolar concentrations of Mg^{2+}, in a voltage-dependent manner (1–4). We have previously shown (16), and we confirm in the present study, that under our culture conditions NMDA-evoked [^{3}H]DA release is not inhibited by Mg^{2+} (1.2 mM) (but is actually enhanced relative to NMDA-evoked [^{3}H]DA release in the absence of Mg^{2+}). However, when ongoing electrical activity in the cultures is dampened by lidocaine or tetrodotoxin, the NMDA response does become Mg^{2+} sensitive (16). An explanation for the enhancement of the NMDA response in the presence of 1.2 mM Mg^{2+} (and absence of tetrodotoxin or lidocaine) remains elusive but, speculatively, may be related to the maturational state of cell cultures, as described for cortical cell cultures by Frandsen *et al.* (37), or to protection by Mg^{2+} against receptor desensitization induced by tonic stimulation of NMDA receptors. The critical observation with respect to the present study is that the NMDA receptor modulating [^{3}H]DA release is sensitive to the blocking effect of Mg^{2+} but that tonic depolarization of the cell cultures overcomes Mg^{2+} blockade of the NMDA response.

When tested in the absence of Mg^{2+}, glycine failed to inhibit the NMDA response. This observation indicates that the inhibition by glycine (in the presence of Mg^{2+}) was probably not mediated by a direct interaction of glycine with the NMDA receptor. Rather, it is consistent with the suggestion that, when 1.2 mM Mg^{2+} is present, glycine hyperpolarizes NMDA-responsive neurons through activation of strychnine-sensitive glycine receptors, allowing Mg^{2+} blockade of the NMDA response. It appears that, if the mechanism by which Mg^{2+} blocks the NMDA response is eliminated (e.g., by performance of the experiment in the absence of Mg^{2+}), inhibitory effects of glycine on the NMDA response are lost. In the intact brain, it is possible that convergent depolarizing inputs onto NMDA-responsive neurons may, under certain conditions, sufficiently counteract the hyperpolarizing effects of glycine so that the voltage-dependent Mg^{2+} blockade, and hence the inhibitory effects of glycine, may be overcome. With alleviation of the Mg^{2+} blockade, the action of glycine at the strychnine-insensitive site would become evident. Thus, in the presence of glycine, the NMDA response may vary from a completely inhibited response to a glycine-potentiated response, depending on the presence or absence of other depolarizing inputs that may modulate the Mg^{2+} blockade.

In the absence of Mg^{2+}, glycine did not inhibit but enhanced NMDA-evoked [^{3}H]DA release, through a strychnine-insensitive mechanism. This suggests that the NMDA receptor stimulating [^{3}H]DA release is associated with a strychnine-insensitive glycine site, capable of potentiating the NMDA response. Consistent with this notion and as previously reported in a similar experiment that tested the effect of kynurenate on NMDA-stimulated [^{3}H]DA release from striatal slices (18), we observed that 7-chlorokynurenate antagonized NMDA-evoked [^{3}H]DA release, in the absence of exogenous glycine, and that the 7-chlorokynurenate antagonism was reversed by addition of glycine (both in the presence and in the absence of 1.2 mM Mg^{2+}). It has been proposed that the potentiating action of glycine at the strychnine-insensitive allosteric site on the NMDA receptor may be an absolute requirement for NMDA receptor activation (38, 39). Thus, in the absence of exogenous glycine, 7-chlorokynurenate presumably inhibits NMDA-evoked [^{3}H]DA release by antagonizing the action of endogenously released glycine at this site. Both spontaneous and EAA-evoked release of endogenous glycine have been observed from primary cultures of striatal neurons (40). It is conceivable that mesencephalic cells also release glycine at levels sufficient to allow NMDA receptor-mediated [^{3}H]DA release and blockade of this release by 7-chlorokynurenate. Glycine appears to be a more potent agonist at the NMDA receptor-associated site than at the strychnine-sensitive glycine receptor, in this system. These results suggest that the tonic effect of glycine in this system is to potentiate the NMDA response. Stimulation of glycine release may be required to elicit the strychnine-sensitive effect of glycine-induced inhibition of the NMDA response. This hypothesis may explain the failure of strychnine alone to potentiate NMDA-evoked [^{3}H]DA release in the presence or absence of exogenous Mg^{2+}.

Strychnine by itself inhibited the NMDA-evoked [^{3}H]DA release from cell cultures, at a concentration of strychnine (100 μM) much higher than that needed to block the strychnine-

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sensitive glycine receptor (1 μM). Araneda and Bustos (9) have recently reported that NMDA-evoked [^{3}H]DA release from substantia nigra slices was both attenuated by 10 μM strychnine and tetrodotoxin sensitive. They suggested, on this basis, that the DA-releasing effect of NMDA in the substantia nigra may be mediated via a trans-synaptic mechanism involving glycinergic neurons. However, in mesencephalic cell cultures, we have shown that NMDA-induced [^{3}H]DA release is not tetrodotoxin sensitive when tested in the absence of exogenous Mg^{2+} (16). Thus, in the present study, strychnine sensitivity of NMDA-evoked [^{3}H]DA release probably does not indicate involvement of glycinergic interneurons but may instead be related to the voltage-dependent blockade of NMDA-activated cationic channels produced by higher concentrations (20 to 60 μM) of strychnine (41). The inhibition of K^+-stimulated [^{3}H]DA release by 100 μM strychnine indicates that, whereas high concentrations of strychnine may not interact with other EAA receptor subtypes, they do not interact selectively with the NMDA receptor.

In summary, the present data indicate that both strychnine-sensitive and strychnine-insensitive glycine receptors are present on rat mesencephalic cells in culture. Strychnine-sensitive glycine receptors inhibit NMDA-stimulated [^{3}H]DA release, whereas strychnine-insensitive glycine receptors potentiate NMDA-evoked [^{3}H]DA release. It has previously been observed that the ability for Mg^{2+} to produce a voltage-dependent blockade of the NMDA receptor allows the NMDA receptor to act as an "input-sensitive amplifier of excitatory synaptic responses" (i.e., a small depolarizing input may alleviate the Mg^{2+} blockade so as to permit activation of an NMDA response) (42). We suggest that, in a system regulated by both inhibitory and potentiating glycine receptors, the dual influence of glycine could allow for even further input-sensitive amplification of the NMDA response.

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Send reprint requests to: Patricia Boksa, Ph.D., Douglas Hospital Research Center, 6875 LaSalle Blvd., Verdun, Quebec, H4H 1R3, Canada.

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