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ABSENCE OF IMPLICATION OF L-ARGININE/NITRIC OXIDE PATHWAY ON NEURONAL CELL INJURY INDUCED BY L-GLUTAMATE OR HYPOXIA+

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L-glutamate, N-methyl-D-aspartate (NMDA), kainate, quisqualate and sodium nitroprusside increased cyclic GMP (cGMP) level on rat whole brain cell culture. The accumulation of cGMP evoked by L-glutamate was inhibited by a NMDA antagonist MK-801, an inhibitor of guanylate cyclase methylene blue and two nitric oxide (NO) synthase inhibitors N^G-monomethyl-L-arginine (L-NMMA) and L-N^G-nitroarginine (NO_2Arg). The inhibition of L-NMMA on cGMP level was reversed partially by addition of L-arginine. Although MK-801 was able to protect cells from neuronal injury induced by L-glutamate or by 5 h hypoxia, L-NMMA and NO_2Arg were ineffective. The present study suggests that cGMP elevation mediated by NO following activation by L-glutamate is not involved in neuronal cell injury.

Endogenous excitatory amino acids (EAAs), L-glutamate and related compounds, are involved in many physiological events in the central nervous system ranging from synaptic plasticity during learning and memory to neurodegenerative diseases including those associated to hypoxic or ischemic neuronal injury. Toxicity of EAAs on neurons has been established both in vivo (1) and in vitro (2) although the mechanisms of neurotoxicity are still a subject of controversy. The effects of L-glutamate are mediated through multiple subclasses of specific receptors divided on N-methyl-D-Aspartate (NMDA) receptors and

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Abbreviations :
cGMP, cyclic guanosine monophosphate ; EAA, excitatory amino acid, NMDA, N-methyl-D-aspartate ; NO, nitric oxide ; L-NMMA, N-L^G-Monomethylarginine ; L-N^G-nitroarginine, NO_2Arg, IBMX, isobutylmethylxanthine ; DMEM, Dulbecco's modified Eagle's medium ; LDH, lactate dehydrogenase.

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non-NMDA receptors: quisqualate and kainate receptors (3,4). The NMDA receptors which are well characterized are selectively inhibited by MK-801, a non competitive antagonist.

In cell suspensions or in slices from rat cerebellum (5-7), in slices from rat hippocampus (8) or in vivo in adult mouse cerebellum (9), nitric oxide (NO) is formed enzymatically in response to activation of EAA receptors. NO is released in a Ca^{++}-dependent manner and activates a soluble guanylate cyclase leading to an increase of intracellular cyclic GMP (cGMP). Generation of NO occurs from the convertion of L-arginine to citrulline by a NO synthase enzyme which has been recently cloned from rat brain (10). It is inhibited by L-arginine analogs such as N^G-monomethyl-L-arginine (L-NMMA) (11) or L-N^G-nitroarginine (NO_2Arg) (12). Interestingly the latter substance has been demonstrated to irreversibly block NO synthase in brain (12). However, whereas cGMP evoked by NO is functionally linked to vasorelaxation in blood vessels, its role in nervous tissue remains to be determined. Considering the neurotoxicity of L-glutamate, it is unknown whether NO participates or on the contrary serves as a protective feedback mechanism. In favor of this second hypothesis, sodium nitroprusside which spontaneously generates NO prevents cerebellar cells from oxygen radical-induced damage (13).

In order to precise the role of NO, we have investigated in cultured brain cells consisting of mature neuronal and glial cells from whole brain the effects of inhibitors of L-arginine pathway on cGMP responses and on neuronal cell injury induced by L-glutamate and hypoxia.

MATERIALS AND METHODS

Materials L-glutamate, NMDA, kainate, quisqualate and MK-801 were purchased from Research Biochemicals, Inc. (Natik, MA, USA), L-NMMA from Calbiochem (La Jolla, CA, USA), NO_2Arg from Aldrich (Strasbourg, France), L-arginine, isobutylmethylxanthine (IBMX), sodium nitroprusside and methylene blue from Sigma (St Louis, MO, USA), cGMP radioimmunoassay kit from New England Nuclear (Boston, MA, USA), lactate dehydrogenase (LDH) kit from Boehringer Mannheim (Ingelheim, GER).

Cell Cultures Brain cell cultures, consisting of both neuronal and glial cells, were prepared from fetal (days 17-18) rat brains using modification of an established technique (14). Cells were maintained in Primaria (Falcon) 35 mm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5 % fetal calf serum, L-glutamine (2mM), steptomycine (50 μg/ml), penicilline (50 UI/ml) and glucose (21 mM). Five days later, cytosine arabinoside (10^{-5} M) was added for 2-3 days. Cultures were selected for study between 12-14 days.

cGMP measurements Cultures were washed twice with DMEM at 37°C and incubated for 5 min in DMEM containing 2.10^{-4} M IBMX. Then cultures were exposed to L-glutamate, NMDA, kainate, quisqualate, or sodium nitroprusside at 37°C for 5 min, except for time course experiments. Incubation was terminated by double washing with DMEM at 4°C and by addition of 0.1 N HCl (15). cGMP levels were estimated by radioimmunoassay. An

aliquot of the cell suspension was used to assay the content of protein (16). When antagonists and/or L-arginine were tested, cells were preincubated with these agents simultaneously with IBMX for 5 or 30 min forehand. For hypoxia experiments, cGMP level was measured during 10 min after 5 h of hypoxia in presence of IBMX as described above.

L-glutamate and hypoxic exposure Experiments were carried out in serum- and glucose-free culture medium. Cells were preincubated with or without the antagonists for 5 or 30 min before L-glutamate exposure or hypoxia. Cells were exposed to L-glutamate for 5 min, then the medium was replaced by culture medium containing the same concentration of antagonists and cultures put back in the incubator. Cell submitted to hypoxia were transferred in a humidified modular incubator (Flow Laboratories) with 95% N2 and 5 % CO2. After 5 h, dishes were replaced in a normoxic incubator (room air with 5% CO2).

Assessment of cellular injury Neuronal cell injury was assessed 24 h after L-glutamate exposure or hypoxia by examination of the culture under phase-contrast microscopy at 200 to 400 magnification. For quantitative evaluation of neuronal cell damage, the efflux into the culture medium of the cytosolic enzyme LDH from damaged neurons was measured by spectrophotometry (17).

RESULTS

Measurement of cGMP

Figure 1 presents the time-dependent increase of cGMP production in response to exposure of L-glutamate (10^{-4} M) on mixed brain cell culture. The maximal increase of cGMP production was reached at 5 min and maintained at this level up to 10 min. Hence, 5 min incubation was routinely used in our study. A similar accumulation of cGMP was observed with 10^{-4} M NMDA, quisqualate or kainate as well as 10^{-4} M sodium nitroprusside (Figure 2).

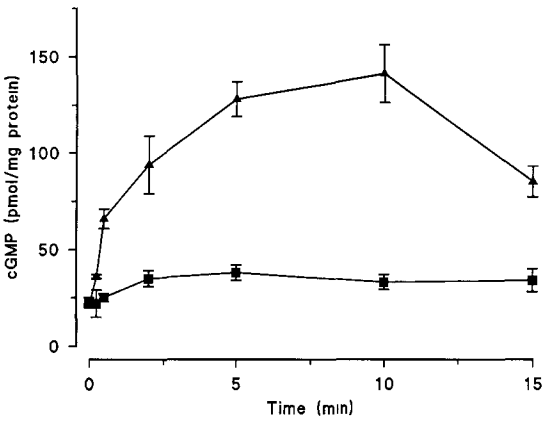

Figure 1 . Time course of cGMP accumulation (pmol/mg protein) following the addition of L-glutamate (10^{-4} M) to brain cell culture. Each point is the mean of triplicate determinations. (■) control, (▲) L-glutamate.

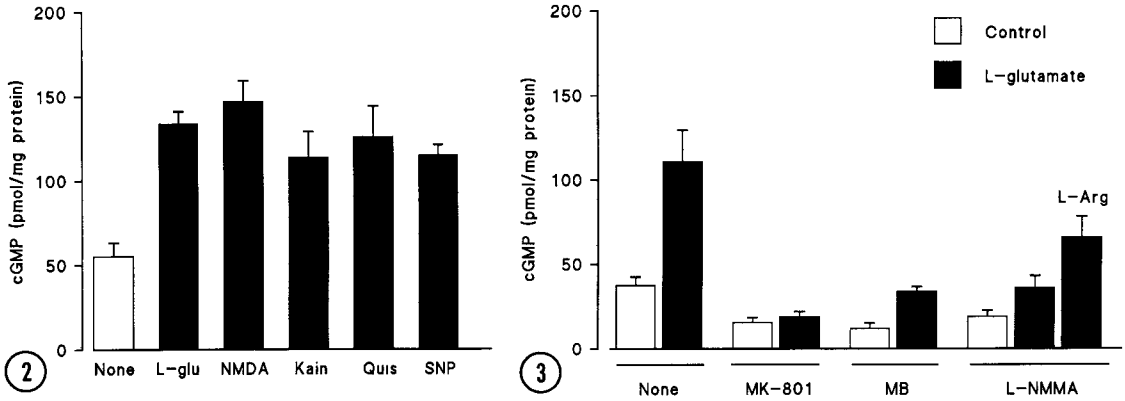

Figure 2. cGMP levels in brain cell culture of rat exposed to 10^{-4} M L-glutamate (L-glu), NMDA, kainate (Kain), quisqualate (Quis) or sodium nitroprusside (SNP). Each point represents the cGMP levels mean ± S.E.M. of 5 separate experiments each done in triplicate.

Figure 3. Inhibition of the cGMP response to L-glutamate in brain cell culture. Cells were preincubated with 10^{-5} M MK-801, 10^{-4} M methylene blue (MB) or 10^{-3} M L-NMMA (with and without L-arginine (L-Arg) at 10^{-3} M) for 5 min before being stimulated for 5 min with 10^{-4} M L-glutamate. Each point represents the mean cGMP levels ± S.E.M. of 3 separate experiments each done in triplicate.

The elevation of cGMP induced by L-glutamate was totally blocked by MK-801 (10^{-5} M), by L-NMMA (10^{-3} M) or by methylene blue (10^{-4} M) as illustrated in Figure 3. Moreover the effect of L-NMMA was partially reversed by L-arginine. A similar inhibitory action on cGMP production was obtained with NO_2Arg (10^{-3} M): cGMP (pmol/mg protein) control, 33 ± 9; L-glutamate, 77 ± 4; NO_2Arg, 3 ± 1; L-glutamate + NO_2Arg, 9 ± 1 (n=3) and by varying the time of preincubation with the inhibitors from 5 min to 30 min. Interestingly it was noted that all the inhibitory substances reduced the basal level of cGMP. In cells submitted to hypoxia the levels of cGMP were considerably diminished: cGMP (pmol/mg protein) control, 50 ± 7, hypoxia, 22 ± 4 (n=3).

Measurement of neuronal cell injury

As shown in Figure 4A representing the cell culture, phase brightness distinguished intact and good shape clusters of neuronal cells with their interconnecting neurites from the underlying glial monolayer. After 24 h following 5 min exposure to L-glutamate (Figure 4B) the neurons were vacuolated, desintegrated leading to debris whereas the glial cells remained almost undisturbed. Addition of MK-801 (10^{-5} M) to the medium completely prevented cells from injury (Figure 4C). In contrast neither L-NMMA (10^{-3} M; Figure 4D) nor NO_2Arg (10^{-3} M) were effective to prevent neuronal cell injury produced by L-glutamate whatever they

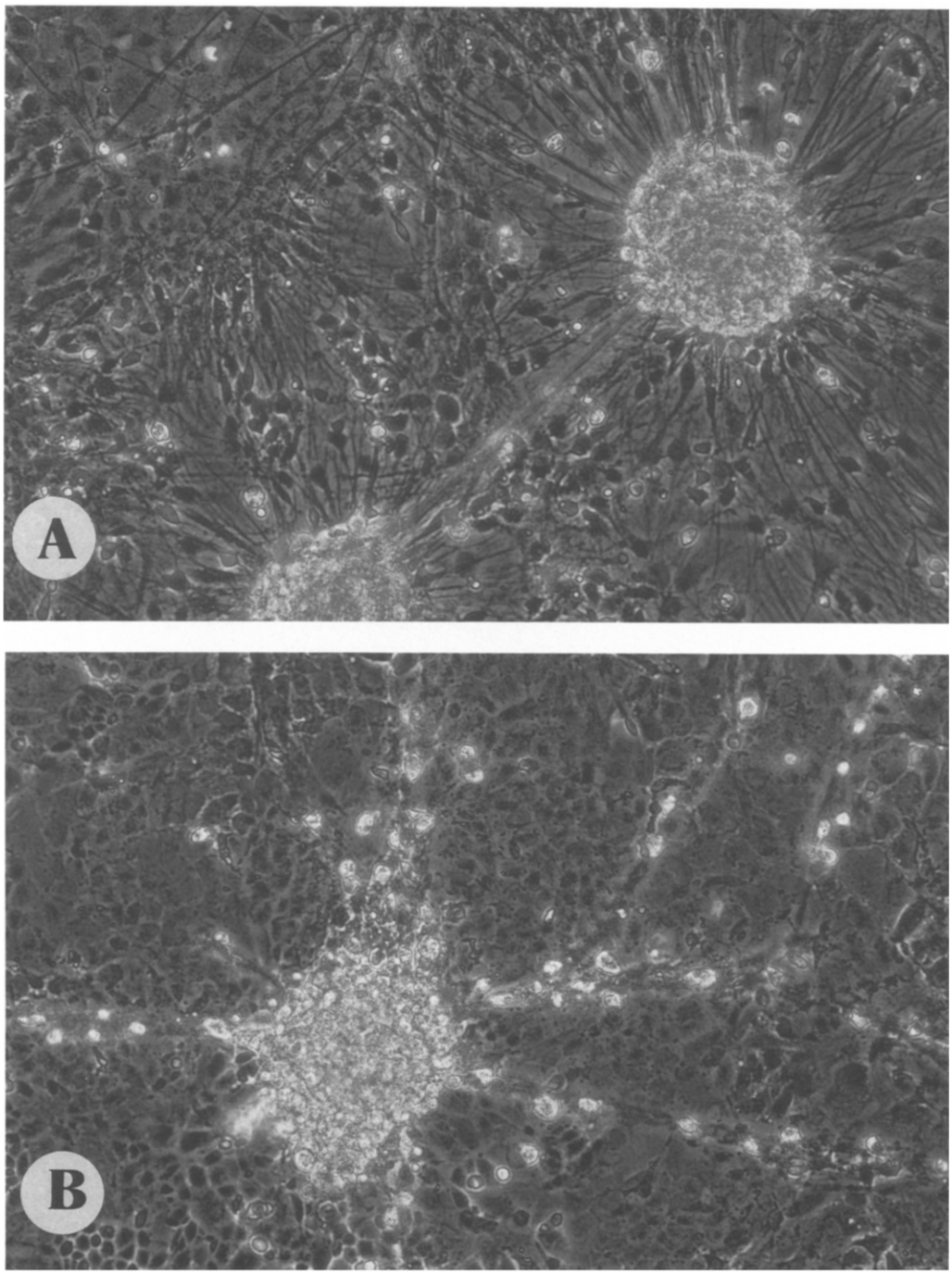

Figure 4 . Effect of L-glutamate on cultured brain cells. Representative fields of neurons were photographed under phase-contrast microscopy. Magnification 180. (A) field just prior to exposure to L-glutamate showing clusters of neuronal cell bodies and interconnecting neurites. (B) after 24 h following 5 min exposure to L-glutamate (10^{-4} M), neuronal cells were disintegrated. (C) field of neurons treated with MK-801 (10^{-5} M) before L-glutamate exposure : the neurons were still intact and processes were not changed. (D) field of neurons treated with L-NMMA (10^{-3} M) before L-glutamate exposure : no protective effect was observed.

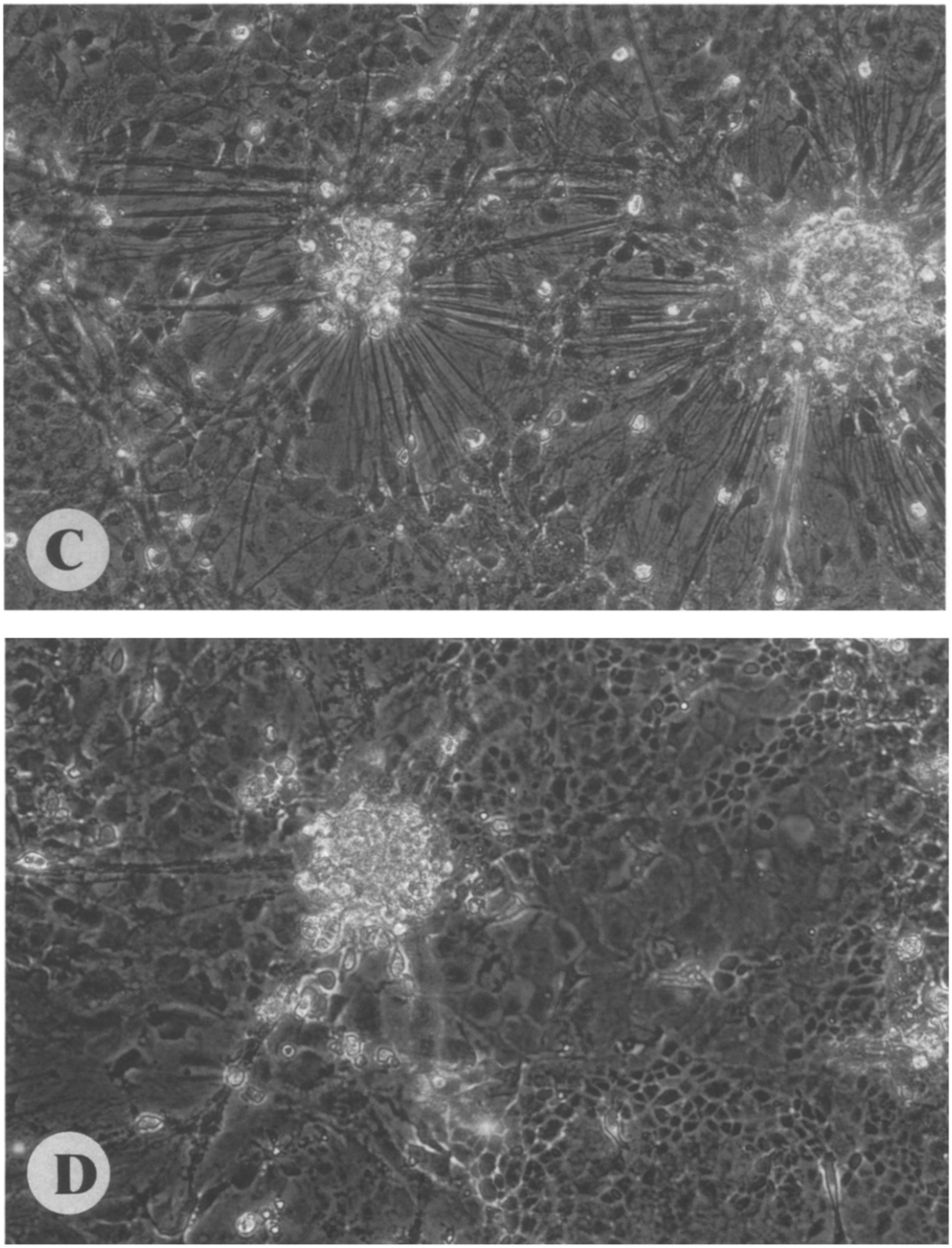

Figure 4 - Continued

were preincubated for 5 or 30 min before the addition of L-glutamate. Identical observations were obtained with these compounds - i.e protection with MK-801, no protection with L-NMMA or NO_2Arg - in cells submitted to 5 h hypoxia. Qualitative morphological

TABLE 1 . Effect of MK-801, L-NMMA and NO_2Arg on neuronal cell injury induced by L-glutamate. Cultured cells were exposed 5 min to L-glutamate (10^{-4} M) and LDH concentration was measured in the bathing medium 24 h later (mean ± SEM values from 3 determinations). Cells were incubated with MK-801 (10^{-5} M), L-NMMA (10^{-3} M) or NO_2Arg (10^{-3} M) 5 min before L-glutamate exposure and during the 24 h period. The data shown are from individual experiment, representative of 3 carried out.

		LDH RELEASE (UI/L)	
Compound		**Control**	**L-glutamate**
None		60 ± 2	122 ± 2
MK-801	(10^{-5} M)	52 ± 12	59 ± 10
L-NMMA	(10^{-3} M)	57 ± 8	137 ± 7
NO_2Arg	(10^{-3} M)	62 ± 6	108 ± 12

assessments of neuronal cell injury were also corroborated by measurement of LDH in the medium 24 h after L-glutamate exposure (Table 1) or hypoxia (Table 2).

DISCUSSION

Although it has been known for many years that EAAs elicit large increase of intracellular cGMP in the central nervous system, it is only recently that Garthwaite et al (5) have demonstrated in rat cerebellum that elevation of cGMP evoked by L-glutamate was coupled to NO release. Our results confirm and extent their observations on mixed cultured brain cells consisting of mature neuronal and glial cells from whole brain. In our model, L-glutamate, NMDA, quisqualate and kainate elevated intracellular cGMP to the same extent than sodium nitroprusside indicating that stimulation of all different types of EAA receptors

TABLE 2 . Effect of MK-801, L-NMMA and NO_2Arg on neuronal cell injury induced by hypoxia. Cultured cells were exposed to 5 h hypoxia and LDH concentration was measured in the bathing medium 24 h later (mean ± SEM values from 3 determinations). Cells were incubated with MK-801 (10^{-5} M), L-NMMA (10^{-3} M) or NO_2Arg (10^{-3} M) 30 min before hypoxia and during the 24 h period. The data shown are from individual experiment, representative of 3 carried out.

		LDH RELEASE (UI/L)	
Compound		**Control**	**Hypoxia**
None		63 ± 7	130 ± 27
MK-801	(10^{-5}M)	57 ± 8	55 ± 8
L-NMMA	(10^{-3} M)	55 ± 9	127 ± 20
NO_2Arg	(10^{-3} M)	53 ± 8	161 ± 18

evokes the formation of cGMP. This has been previously observed in cultured rat cerebellar cells (18) and in vivo in adult mouse cerebellum (9). On the contrary, the dramatic decrease of cGMP concentration observed after 5 h hypoxia may reflect decreased availability of GTP to cGMP synthesis.

The stimulation of cGMP production was inhibited and even lowered under the basal level by MK-801, a selective non competitive NMDA antagonist. Although L-glutamate activates both NMDA and non-NMDA receptors, blockade by MK-801 was sufficient to totally inhibit cGMP elevation. These results are however in accordance with those obtained in cultured cerebellar granule cells (19) and in dissociated cells from developing cerebellum (20) using the competitive NMDA antagonist, 2-amino-5-phosphonovalerate. In part, this may be due to the high affinity of L-glutamate for NMDA receptors (21) and/or a direct or indirect activation of NMDA receptors by kainate and quisqualate (22). Moreover, the effect of MK-801 on the basal level of cGMP suggests a release of endogenous L-glutamate in the medium. On the other hand L-NMMA and NO_2Arg abolished the effect of L-glutamate on cGMP with the same potency than methylene blue and MK-801 showing that it is dependent on the generation of NO as demonstrated on other brain tissues (5-9). As expected (5-7) the effect of L-NMMA was partially reversed by L-arginine. Therefore, it appears that EAA stimulation induced an activation of guanylate cyclase by a NO-dependent mechanism on mixed brain neuronal and glial cells in culture.

To assess the role of NO, we examined by phase-contrast microscopy cellular damage and measured LDH release in the medium after L-glutamate exposure or hypoxia in presence or not of NO synthase inhibitors. Indeed exposure of neuronal glial cells to L-glutamate or hypoxia produced a widespread late neuronal degeneration as described in cortical (23) and hippocampal (2) cell cultures. MK-801 afforded a total protection against neuronal cell injury. This is in agreement with previous reports in cortical cell cultures (24). By contrast, in the same experimental conditions, inhibitors of NO synthase failed to protect against neuronal cell injury. L-NMMA as well as the highly potent and irreversible L-arginine analog NO_2Arg, whatever the preincubation time, did not protect nor enhance the neurotoxic effect of L-glutamate or hypoxia. Since the production of cGMP by L-glutamate was totally blocked by the NO synthase inhibitors without any change on neuronal cell injury, our data suggest that the NO pathway is not involved in their neurotoxicity in whole brain cell culture. These results differ from those of Dawson et al (25) published during the time of writing our paper. These authors have shown that in rat primary cortical cultures L-NMMA or NO_2Arg prevent L-glutamate neurotoxicity. Although more detailed studies will be necessary to precise the role of NO in neuronal cell injury, the discrepancy in the results

might be explained partly by the distinct origin of cultured cells derived from highly vulnerable brain areas in their experiments and from whole brain in our study.

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