Neurochemical Research, Vol. 21, No. 10, 1996, pp. 1237–1244

NMDA Receptor Antagonists Prevent Acute Ammonia Toxicity in Mice

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(Accepted June 18, 1996)

We proposed that acute ammonia toxicity is mediated by activation of NMDA receptors. To confirm this hypothesis we have tested whether different NMDA receptor antagonists, acting on different sites of NMDA receptors, prevent death of mice induced by injection of 14 mmol/Kg of ammonium acetate, a dose that induces death of 95% of mice. MK-801, phencyclidine and ketamine, which block the ion channel of NMDA receptors, prevent death of at least 75% of mice. CPP, AP-5, CGS 19755, and CGP 40116, competitive antagonists acting on the binding site for NMDA, also prevent death of at least 75% of mice. Butanol, ethanol and methanol which block NMDA receptors, also prevent death of mice. There is an excellent correlation between the EC_{50} for preventing ammonia-induced death and the IC_{50} for inhibiting NMDA-induced currents. Acute ammonia toxicity is not prevented by antagonists of kainate/AMPA receptors, of muscarinic or nicotinic acetylcholine receptors or of GABA receptors. Inhibitors of nitric oxide synthase afford partial protection against ammonia toxicity while inhibitors of calcineurin, of glutamine synthetase or antioxidants did not prevent ammonia-induced death of mice. These results strongly support the idea that acute ammonia toxicity is mediated by activation of NMDA receptors.

KEY WORDS: Ammonia toxicity; NMDA receptors; NMDA receptor antagonists; glutamate neurotoxicity.

INTRODUCTION

Hepatic encephalopathy is one of the main causes of death in occidental countries. However, its pathogenesis is not well understood and several hypotheses have been proposed as explanations for its effects. Hyperammonemia is considered one of the main factors responsible for hepatic encephalopathy. Increased ammonia levels accompany a number of human diseases, including liver cirrhosis and fulminant hepatic failure. Elevated blood ammonia levels induces toxic effects, with functional disturbances in the central nervous system. It is also known that injection into animals of large doses of ammonia leads to death of animals.

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ABBREVIATIONS: AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; AP-5: 2-amino-5-phosphonovaleric acid; CPP: 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; CGP 40116: D-E-4-(3-phosphonoprop-2-enyl)piperazine-2-carboxylic acid; CGS 19755: cis-4-phosphonomethyl-2-piperidine carboxylic acid; DNQX: 6,7-dinitroquinoxaline-2,3-dione; Ketamine: 2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone; Memantine: 3,5-Dimethyl-1-adamantan-amine; MK-801: (5S, 10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5, 10-imine hydrogen maleate; NAME: nitroarginine methyl ester; NBQX: 6-nitro-7-sulfamoyl-benzo(F)quinoxaline-2,3-dione; NO: nitric oxide; NOS: nitric oxide synthase; PBN: N-tert-butyl-α-phenylnitrone; PCP: 1-[1-phenyl-cyclohexyl]-piperidine.

0364-3190/96/1000-1237$09.50/0

The mechanism by which ammonia causes these deletereous effects has not been clarified. A possible role for excitatory neurotoxic amino acids has been suggested by Moroni et al. (1–4). Acute ammonia intoxication leads to increased release of glutamate from the brain surface of the rat (1). Moreover, the content of quinolinic acid, an excitotoxic compound, is increased in animal models of hepatic encephalopathy (4) and in cerebrospinal fluid and frontal cortex from patients with hepatic failure (3). In agreement with this, we have recently shown that MK-801, an antagonist of the NMDA type of glutamate receptors, prevents death of animals injected with large doses of ammonium acetate (5), suggesting that acute ammonia toxicity is mediated by activation of NMDA receptors.

The aim of the present work was to confirm this hypothesis by testing the possible protection against acute ammonia toxicity afforded by different NMDA receptor antagonists acting on different sites of the receptor (non competitive and competitive antagonists).

Glutamate, when in excess, is neurotoxic. In many systems, glutamate neurotoxicity is mediated by activation of NMDA receptors (6,7) and concomitant increase in intracellular Ca^{2+}. However, the subsequent steps leading to neuronal death have not been clarified. The molecular mechanism of glutamate neurotoxicity is now being extensively studied and several possible mediators have been proposed, including activation of nitric oxide synthase, of calcineurin or formation of free radicals. To further clarify the molecular mechanism of ammonia toxicity, we have also tested whether interfering with these processes prevents acute ammonia toxicity in mice.

EXPERIMENTAL PROCEDURE

Male Swiss mice weighing 25–35 g were used. Except when indicated, compounds were injected intraperitoneally 15 min before ammonium injection. Ammonium acetate (14 mmol/Kg) was also injected intraperitoneally. The concentrations of the solutions were adjusted to reach the desired dose by injecting 3 µl per g of body weight. The compounds tested and their effects on receptors, enzymes or systems are summarized in Table I.

It has been shown that antioxidants are only effective after continued administration for several days. Therefore, to test the possible protective effects of lipoic acid, α-tocopherol and acetylcysteine these compounds were administered chronically as follows: lipoic acid was administered during 18 days by i.p. injection, 5 times per week, of 100 mg/Kg of body weight as described by Nagamatsu et al. (8). α-Tocopherol was administered during 18 days by i.p. injection, 3 times per week, of 200 mg/Kg as described by Nickander et al. (9). Acetylcysteine was administered during 18 days in the drinking water (10 mg of acetylcysteine/ml) as indicated by Ornaghi et al. (10).

Determination of Cyclic GMP in Brain. Male Wistar rats weighing 250–270 g were injected with ammonium acetate (7 mmol/Kg of body weight) or with saline. Fifteen min later rats were killed by decapitation and brains were immediately freeze-clamped. Tissue was powdered under liquid nitrogen and homogenized in 2.5 ml/g tissue of 50 mM Tris-HCl, 4 mM EDTA, pH 7.5. Homogenates were heated at 100°C for 3 min and centrifuged at 12,000 *g* and 4°C, 10 min. Cyclic GMP was determined in the supernatants using the cyclic GMP [^{3}H]assay system from Amersham.

Assay of Aconitase Activity in Brain. Rats were injected with saline or ammonium acetate as above and killed fifteen min later. Brains were homogenized in 6 ml/g tissue of 250 mM sucrose, 20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 2 mM KH_2PO_4, 1 mM EGTA. Homogenates were centrifuged (640 *g*, 4°C, 10 min). The supernatant was centrifuged again (7000 *g*, 4°C, 10 min) and the pellet was lysed by homogenization in 0.2% Triton X-100, 30 mM NaCl in 30 mM Tris-HCl, pH 7.4. Samples were centrifuged and the supernatant was immediately assayed for aconitase activity as described by Corbett et al. (11).

Materials. AMPA, AP-5, CPP, ketamine, MK-801 and PCP were obtained from Research Biochemicals International, Natick, MA, U.S.A. CGP 40116 and CGS 19755 were generous gifts of Ciba Geigy in Barcelona, Spain. DNQX was from Tocris Cookson, Bristol, UK. Memantine and NAME were from Sigma Chemical Co. St. Louis, MO, U.S.A. PBN was from Molecular Probes, Eugene, OR, U.S.A.

The experimental protocols have been approved by the Scientific Committee of the Institute and meet the guidelines of the European Union for Care and Use of Laboratory Animals.

RESULTS AND DISCUSSION

We tested the effect of ketamine and phencyclidine, compounds acting as channel blockers at the NMDA receptor. As shown in Table II, 75% of mice injected with 50 mg/Kg of ketamine survived following injection of ammonia while only 5% of animals injected only with ammonia survived. Injection of 25 mg/Kg of PCP also protected 78% of the animals. Memantine also prevents ammonia toxicity although it is less efficient than the other channel blockers tested.

These results confirm that agents blocking the ion channel of NMDA receptors prevent nearly completely ammonium-induced death of mice. We then tested whether competitive antagonists of these receptors, acting on the binding site for glutamate and NMDA are also able to prevent ammonia toxicity. As shown in Table II, injection of 20 mg/kg of CPP protects 77% of animals against acute ammonia toxicity. AP-5 also protects mice but the dose required is higher and the number of animals used was low because the amounts of AP-5 required were very expensive. CGS 19755 and CGP 40116, which also act as competitive antagonists of NMDA receptors protect 83% and 75% of mice, respectively, against ammonia toxicity. These results confirm that competitive antagonists of NMDA receptors also prevent ammonia-induced death of mice.

It has been reported that methanol, ethanol and butanol inhibit NMDA-activated currents in cultured hip-

Table I. List of Compounds Injected and of Their Actions on Receptors, Enzymes, or Systems

Compound	Action and Reference	
MK-801	NMDA receptor antagonist. Non competitive Channel blocker.	Wong et al, 1988; 14
Ketamine	NMDA receptor antagonist. Non competitive Channel blocker.	Anis et al, 1983; 45
PCP	NMDA receptor antagonist. Channel blocker.	Anis et al, 1983; 45
Memantine	NMDA receptor antagonist. Channel blocker.	Chen et al, 1992; 46
CGP 40116	NMDA receptor antagonist. Competitive.	Fagg et al, 1990; 47
CGS 19755	NMDA receptor antagonist. Competitive.	Murphy et al, 1988; 48
AP-5	NMDA receptor antagonist. Competitive.	Davies et al, 1980; 49
CPP	NMDA receptor antagonist. Competitive.	Davies et al, 1986; 50
Ethanol	NMDA receptor antagonist.	Weight et al, 1991; 15
Methanol	NMDA receptor antagonist.	Weight et al, 1991; 15
Butanol	NMDA receptor antagonist.	Weight et al, 1991; 15
NBQX	AMPA receptor antagonist.	Sheardown et al, 1990; 51
DNQX	Kainate/AMPA receptors antagonist.	Honoré et al, 1988; 52
Atropine	Muscarinic acetylcholine receptors antagonist.	Rossum, 1960; 53
Tubocurarine	Nicotinic acetylcholine receptors antagonist.	Pedersen and Cohen, 1990; 54
Bicuculline	$GABA_A$ receptors antagonist.	Johnston, 1978; 55
Nitroarginine	Nitric oxide synthase inhibitor.	Moore et al, 1990; 57
NAME	Nitric oxide synthase inhibitor.	Rees et al, 1990; 58
Cyclosporin	Calcineurin inhibitor.	Liu et al, 1991; 59
FK-506	Calcineurin inhibitor.	Liu et al, 1991; 59
Trifluoperazine	Calcineurin inhibitor.	Stewart et al, 1983; 60
Meth. sulfox.	Glutamine synthetase inhibitor.	Tate et al, 1972; 61
PBN	Antioxidant, spin trapper.	Saprin and Piette, 1977; 62
Lipoic acid	Antioxidant.	Suzuki et al, 1991; 63
α-tocopherol	Antioxidant, free radical trapping.	Riely et al, 1974; 64
Acetylcysteine	Antioxidant.	Ornaghi et al, 1993; 10
Mannitol	Antioxidant.	Halliwell and Gutteridge, 1985; 65
Allopurinol	Xantine oxidase inhibitor.	Massey et al, 1970; 66

pocampal neurons (12). The mechanism by which these alcohols inhibit NMDA-activated currents is not clear, however, they do not act by blocking the ion channel or by competing with the agonist (12). It is possible that they interfere with the action of glycine, which has a different binding site in the NMDA receptor (16,17). In any case, alcohols interfere with NMDA receptors at a different site than MK-801, ketamine, PCP, CPP, CGS 19755, CGP 40116, or AP-5. It has been previously shown that these alcohols also prevent acute ammonia toxicity in mice (18), although the molecular mechanism involved remained unclear. We suspected that alcohols prevent ammonia toxicity by inhibiting activation of NMDA receptors. To assess this possibility we tested whether there is a correlation between the ability of the alcohols to inhibit NMDA receptor activation and their capacity to prevent ammonia toxicity. The IC_{50} for inhibition of NMDA-activated currents in cultured hippocampal neurons for each of the alcohols are: methanol $\approx$ 117 mM, ethanol $\approx$ 30 mM and butanol $\approx$ 1.14 mM (12). We determined the doses of these alcohols that prevent death of 50% of mice injected with ammonia (EC_{50}). These doses were 4, $\approx$29 and $\approx$90 mmol/Kg for butanol, ethanol and methanol, respectively. As shown in Fig. 1, there is an excellent correlation between the capacity of these alcohols to inhibit NMDA-induced currents and that to prevent ammonia-induced death of mice, suggesting that their protective effect against ammonia toxicity is due to their ability to block NMDA receptors.

We have previously found that MK-80, which blocks the ion channel of the NMDA receptor (13), prevents death of mice and rats injected with lethal doses of ammonium acetate (5). The protective effect of MK-801 has been later confirmed by Seiler et al. (14) but not by Itzhak and Norenberg (15). A possible explanation for the lack of effect found by Itzhak and Norenberg could be the fact that they inject MK-801 30 min before ammonia while Marcaida et al. or Seiler et al. inject MK-801 10 or 15 min before ammonia.

The above results show that ten different compounds, acting on at least three different sites of NMDA receptors are able to prevent nearly completely ammonia-induced death of mice. This strongly supports the idea that acute ammonia toxicity is mediated by activation of NMDA receptors.

Table II. Effects of Different Compounds on Ammonia-Induced Death of Mice

Compound	Dose mg/kg or mmol/Kg (a	Survival Survivors/ injected	Death %
NONE	—	10/218	95
MK-801	2	21/ 30	25
Ketamine	20	9/ 20	55
Ketamine	50	32/ 43	25
PCP	25	25/ 32	22
Memantine	30	11/ 32	66
CGP 40116	50	6/ 10	40
CGP 40116	100	15/ 20	25
CGS 19755	20	20/ 24	17
AP-5	150	6/ 10	40
AP-5	500	4/ 5	20
CPP	20	23/ 30	23
Ethanol	20	13/ 30	57
Ethanol	50	15/ 21	29
Methanol	90	11/ 20	45
Butanol	2	2/ 10	80
Butanol	4	10/ 20	50
Butanol	6	10/ 10	0
NBQX	30	9/ 20	55
DNQX	25	0/ 10	100
Atropine	0.1	5/ 20	75
Tubocurarine	0.2	7/ 28	75
Bicuculline	7	1/ 10	90
Bicuculline	20	0/ 10	100
Nitroarginine	30	9/ 30	72
Nitroarginine	45	20/ 42	52
NAME	75	12/ 34	68
Cyclosporin	50	0/ 8	100
FK-506	10	1/ 12	92
Trifluoperazine	10	1/ 7	86
Methionine sulfoximine	100	0/ 10	100
PBN	150	0/ 20	100
Lipoic acid	*	0/ 10	100
α-tocopherol	*	2/ 10	80
Acetylcysteine	*	0/ 12	100
Mannitol	1000	1/ 20	95
Allopurinol	200(b	4/ 22	82

Groups of mice were injected with the indicated doses of each compound. Except when indicated, compounds were injected intraperitoneally 15 min before injecting 14 mmol/Kg of ammonium acetate. The number of animals injected and of those surviving after 24 hours are indicated. Mice injected only with ammonia died between 5 and 15 min after injection.

a = All doses are given in mg/Kg except for butanol, ethanol and methanol, which are given in mmol/Kg. These alcohols were injected 5 min before ammonia; b = allopurinol was injected subcutaneously 75 min before ammonium injection.

*Lipoic acid, α-tocopherol and acetylcysteine were administered chronically during 18 days as described in Experimental Procedure.

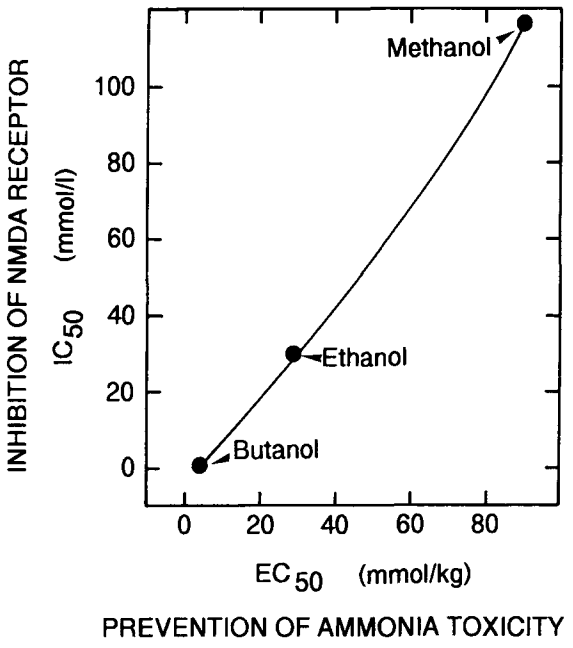

Fig. 1. Relation between potency of different alcohols for inhibiting NMDA-activated ion current and potency of the alcohols for protecting against ammonia toxicity. Groups of mice were injected i.p. with different concentrations of the alcohols 5 min before injecting 14 mmol/Kg of ammonium acetate. The number of animals surviving for each dose is given in Table II. From these values the dose of each alcohol that prevents death of 50% of mice (EC_{50}) was calculated. The values for IC_{50} for inhibition of NMDA-induced currents were taken from Weight et al. (15).

We then tested the possible protective effect of antagonists of other types of glutamate receptors. As shown in Table II, injection of 30 mg/Kg of NBQX, an antagonist of AMPA receptors, prevents partially ammonia toxicity in mice. DNQX, an antagonist of kainate/AMPA receptors, did not prevent at all ammonia toxicity in mice. These results suggest that acute ammonia toxicity is mainly mediated by activation of NMDA receptors and not of other glutamate receptors.

To assess the possible effect of other neurotransmitters in the mediation of ammonia toxicity, we tested whether antagonists of muscarinic or nicotinic acetylcholine receptors or of GABA receptors are able to prevent ammonia toxicity. As shown in Table 2, atropine, tubocurarine or bicuculline, antagonists of these receptors did not prevent ammonia toxicity, suggesting that these receptors do not play an important role in the mediation of its toxicity.

The above results indicate that acute ammonia toxicity is mediated by activation of NMDA receptors. It is well known that glutamate, when in excess, is neurotoxic and in most systems, its neurotoxicity is mediated by activation of NMDA receptors (6,7). It seems therefore likely that, following activation of NMDA receptors, the molecular mechanisms of ammonia toxicity and of glu-

tamate neurotoxicity would be the same. The molecular mechanism of glutamate neurotoxicity is now being extensively studied but is not clearly understood. Activation of NMDA receptors leads to opening of the associated ion channel, allowing the entry into the neuron of Na^+ and Ca^{2+}. The rise in intracellular Ca^{2+} seems to be an essential step in the process leading to neuronal death (19,20). However, the subsequent steps in this process have not been clearly identified. The rise in Ca^{2+} activates different Ca^{2+}-dependent enzymes, including nitric oxide synthase (21,22) and the protein phosphatase calcineurin (23). It has been shown that inhibitors of these enzymes prevent glutamate neurotoxicity in primary cultures of neurons (23–26). However, in other systems, inhibition of nitric oxide did not prevent glutamate neurotoxicity (27,28). We therefore tested whether inhibitors of nitric oxide synthase (nitroarginine or nitroarginine methyl ester) or of calcineurin (cyclosporin, FK-506 or trifluoperazine), are able to prevent acute ammonia toxicity in mice. Inhibitors of nitric oxide synthase afford partial protection against ammonia toxicity (Table 2 and Kosenko et al. (29)). None of the inhibitors of calcineurin tested protected mice against ammonia toxicity (Table II).

Excessive activation of glutamate receptors results in the formation of free radicals by different ways (30–32). It has been suggested that these free radicals and the associated oxidative stress would be involved in the process leading to neuronal death (33,34). The rise of intracellular Ca^{2+} following activation of NMDA receptors activates xanthine oxidase, which generates free radicals (35). As shown in Table II, allopurinol, an inhibitor of xanthine oxidase did not prevent acute ammonia toxicity in mice. We also tested the effect of other antioxidants administered chronically for 18 days before ammonia injection. As shown in Table 2, chronic administration of lipoic acid, α-tocopherol or N-acetylcysteine is unable to afford protection against ammonia toxicity. Acute administration of PBN, a spin trapper, or of mannitol, another antioxidant, were also ineffective in preventing ammonia toxicity. Therefore none of the antioxidant agents tested were able to prevent ammonia-induced death of mice. It should be noted that the doses and times of administration of the compounds used are enough to have the desired effect as antioxidants. Protective effects against different injuries have been reported, using the same conditions of administration, for lipoic acid (8), α-tocopherol (9), acetylcysteine (10), PBN (36), mannitol (37) and allopurinol (38). It seems therefore that antioxidants are not able to prevent acute ammonia toxicity in mice.

Finally, it has been suggested that glutamine synthesis is an essential step in the mediation of ammonia toxicity (39,40). As shown in Table II, injection of 100 mg/Kg of methionine sulfoxime, an inhibitor of glutamine synthetase, did not prevent ammonia toxicity. This indicates that glutamine synthesis is not required for ammonia toxicity. This is in agreement with our previous results showing that both MK-801 and nitroarginine increased significantly glutamine content in brain while preventing ammonia-induced death of mice (29,41), indicating that increased glutamine is not responsible for ammonia toxicity.

The above results show that ten different NMDA receptor antagonists, acting on at least three different sites of the receptor are able to prevent nearly completely ammonia-induced death of mice. This strongly supports the idea that acute ammonia toxicity is mediated by activation of NMDA receptors. Antagonists of other glutamate receptors, of muscarinic or nicotinic acetylcholine receptors or of GABA receptors did not prevent ammonia toxicity. Inhibitors of nitric oxide synthase (NOS) afford partial protection against ammonia toxicity, suggesting that following activation of NMDA receptors, there is an activation of NOS, which would be involved in the process leading to animal death. To confirm that ammonium injection leads to increased nitric oxide (NO) in brain, we determined two parameters which are used as indirect measure of nitric oxide: cyclic GMP and activity of aconitase.

Guanylate cyclase is stimulated by NO and increased NO formation is associated with increased cGMP content (42,43). We determined cGMP in brains from control rats or from rats injected with ammonia. For controls cGMP content was 25 ± 3 pmol/g tissue. Fifteen min after injection of ammonia, the brain content of cGMP was 27 ± 3 pmol/g tissue (Table 3). Another parameter that reflects NO levels is the activity of aconitase. Aconitase is inhibited by NO (44) and increased NO is associated with decreased aconitase activity. For control rats, aconitase activity in brain was 43 ± 5 nmol/mg protein while for rats injected with ammonia it was 44 ± 5 nmol/mg protein (Table III). These results suggest that the increase in brain NO following ammonia injection would be very slight, if any. The protective effect of NOS inhibitors could be explained because formation of NO mediates ammonia toxicity or, alternatively, it could be due to inhibition of NOS in endothelial cells. It is well known that NO produced by these cells modulates cerebral blood flow. NOS inhibitors could decrease cerebral blood flow and this effect could influence ammonia toxicity. Another possibility is

Table III. Effect of Acute Ammonia Intoxication on cGMP Content and on Aconitase Activity in Rat Brain

	cGMP	Aconitase activity
Ammonia injection	pmol/g tissue	nmol/mg prot. min
No	25 ± 3	43 ± 5
Yes	27 ± 3	44 ± 5

Male Wistar rats weighing 250-270 g were injected i.p. with 7 mmol/kg of ammonium acetate or with saline. Fifteen min later rats were killed by decapitation and the brains were immediately removed. Cyclic GMP and aconitase activity were determined as indicated in Materials and Methods. Values are the mean ± standard deviations of the values obtained for eight rats per group.

that arginine derivatives reduce ammonia toxicity through an unknown mechanism independent from NO.

As shown in Table II, three different inhibitors of calcineurin and six different antioxidants, with different mechanisms of action, were unable to afford any protection against ammonia toxicity.

Although there are not detailed studies about the blood-brain barrier permeability of most of the compounds used, most of them have been previously used at similar concentrations in animals. For some of them it has been shown to reach the cerebrospinal fluid, e.g. memantine (67) while for many others protective effects in cerebral ischemia have been reported, e.g. for DNQX (51), CGS 19755 (68), PBN (69), CGP 40116 (70), indicating that they can reach the brain. Other reports also indicate that ketamine (71). phencyclidine (72) and methionine sulfoximine (39) can reach the brain. For antioxidants, protective effects against different injuries have been reported, using the same conditions of administration, for lipoic acid (8), α-tocopherol (9), acetylcysteine (10), PBN (36), mannitol (37) and allopurinol (38).

The results reported indicate that NMDA receptor antagonists reduce acute ammonia toxicity, supporting the idea that acute ammonia toxicity is mediated by activation of NMDA receptors. The subsequent events leading to animal death remain to be clarified. There are at least two mechanism by which ammonia could lead to increased activation of NMDA receptors: by increasing extracellular glutamate content in brain or by modulating the function of NMDA receptors. Increased extracellular glutamate in brain following acute ammonia intoxication has been described by Moroni et al. (1) and confirmed by DeKnegt et al. (73). Increased glutamate concentration could be due to increased release or to decreased uptake of glutamate by astrocytes. Both effects have been described in the literature (74–76). Ammonia is considered one of the main factors in the mediation of hepatic encephalopathy and, possibly, of hepatic coma. If this is the case, NMDA receptor antagonists could have potential therapeutic utility in the clinical treatment of hepatic coma.

ACKNOWLEDGMENTS

FK-506 and cyclosporin were generous gifts from Fujisawa Gmbh and Sandoz Pharma S.A.E., respectively. CGS 19755 and CGP 40116 were generous gifts from Ciba Geigy. This work was supported in part by grant 93/0187 from the Fondo de Investigaciones Sanitarias of Spain. G. Marcaida received a fellowships from the Generalitat Valenciana. C. Hermenegildo received the "Severo Ochoa" fellowship from the Ayuntamiento de Valencia.

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