J Neural Transm [GenSect] (1994) 97: 187–196

Journal of Neural Transmission

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Printed in Austria

Ammonium acetate inhibits ionotropic receptors and differentially affects metabotropic receptors for glutamate

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Accepted April 28, 1994

Summary. The effects of ammonium salts in concentration similar to those found in plasma in course of hepatic encephalopathy (2–4 mM) were studied in brain slices in order to clarify how glutamate synapses are affected by this pathological situation. Electrophysiological (mice cortical wedge preparations) and biochemical techniques (inositol phosphates and cyclic AMP measurements) were used so that the function of both the ionotropic and metabotropic glutamate receptors was evaluated. Ammonium acetate (2–4 mM), but not sodium acetate reduced the degree of depolarization of cortical wedges induced by different concentrations of N-methyl-D-aspartic acid (NMDA) or (S)-alpha-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). This reduction was non-competitive in nature and did not reverse during the experimental period (90 min). In a similar manner, ammonium acetate reduced the formation of inositol phosphates induced by (1S,3R)-1-amynocyclopentane-1,3-dicarboxylic acid (1S,3R-ACPD) (100 μM), the prototype agonist of metabotropic glutamate receptors. When the metabotropic glutamate receptors negatively linked to the forskolin-stimulated cyclic AMP formation were evaluated, ammonium acetate significantly hampered forskolin effects and its actions were additive with those of the metabotropic glutamate receptor agonist 1S,3R-ACPD. In conclusion, our results suggest that toxic concentrations of ammonium impair the function of glutamate receptors of NMDA and AMPA type and of the metabotropic glutamate receptors linked to inositol phosphate formation while they functionally potentiate the action of glutamate agonists on the receptors negatively linked to adenylyl cyclase.

Keywords: Ammonia toxicity, glutamate receptors, 1S,3R-ACPD, cyclic AMP, inositol phosphates, hepatic encephalopathy.

Introduction

The administration of ammonium salts, or the accumulation of ammonia due to impaired urea formation, results in signs and symptoms of hepatic enceph-

alopathy which are associated with an increased concentration of glutamate in the brain extracellular spaces (Moroni et al., 1983) and with other modifications of the function of glutamate neurotransmission (Theoret et al., 1985; Fan et al., 1990; Rao et al., 1992). In fact, in several brain areas of the rat, toxic concentrations of ammonia decrease the number of NMDA and AMPA/Kainic (KA) recognition sites (Peterson et al., 1990; Rao et al., 1991; Maddison et al., 1991). Similar concentrations of this ion in the human and rat brain increase the content of quinolinate, another endogenous ligand of glutamate receptors (Moroni et al., 1983, 1986; Tossman et al., 1987) and modify the neosynthesis of the transmitter pool of glutamate, in hippocampal slices (Hamberger et al., 1979; Fan et al., 1990).

We report here that toxic concentrations of ammonium acetate reduce the function of the ionotropic glutamate receptor of NMDA and AMPA type "in vitro". In fact this salt, but not sodium acetate, reduces NMDA or AMPA induced depolarization of mice cortical wedges (Harrison and Simmonds, 1985). Furthermore, in rat hippocampal slices ammonium acetate both reduces the function of metabotropic glutamate receptors (mGluRs) which control the formation of inositol phosphates as well as potentiates the function of mGluRs which modulate the accumulation of cyclic AMP.

Materials and methods

Material

(1S,3R)-ACPD and AMPA were purchased from Tocris Neuramin (Bristol, U.K.). Myo-2-[^{3}H]N-inositol (10–20 Ci/mmol) was from New England Nuclear (Du Pont de Nemours, Milan, Italy); [^{3}H]cyclic AMP radioimmunoassay kit was procured from Amersham (Amity PG, Milan, Italy); KA, forskolin, isobutyl-1-methylxanthine (IBMX), Dowex AG-1-X 8 anion exchange resin (100–200 mesh) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The scintillation fluid (Instagel) was purchased from Packard (Groningen, The Netherlands). All other reagents of analytical grade were obtained from Merck (Darmstadt, Germany).

Mice cortical wedge preparations

The cortical wedge preparation described by Harrison and Simmonds (1985) and modified by Burton et al. (1988) was used as previously described (Moroni et al., 1991; Carlà and Moroni, 1992). Briefly, wedges were placed into a two-compartment bath and silicone grease was placed between the two portions of the bath. The wedges were incubated at room temperature and perfused with Krebs solution: (mM) NaCl 135, $CaCl_2$ 2.4, KH_2PO_4 1.3, $MgCl_2$ 1.2, $NaHCO_3$ 16.3, and glucose 7.7, gassed with 95% O_2 and 5% CO_2 at a flow rate of 2 ml/min. After stabilization the gray matter was superfused with a Mg^{++} free medium and agonists of the excitatory amino acids were repeatedly applied for 2 min every 15 min. The D.C. potential between the two compartments was continuously monitored via $Ag/AgCl_2$ electrodes and displayed on a chart recorder. The preparations were initially stabilized by repeated application of 4 μM AMPA or 10 μM NMDA. The responses to 15 μM NMDA and 10 μM AMPA were considered maximal (100%) and these NMDA or AMPA concentrations were applied when the wedges gave stable responses and before starting the dose-response curves. In preliminary experiments we observed that these con-

centrations of NMDA or AMPA caused, under our experimental conditions, a quasi-maximal response without significant desensitization.

Preparation of rat hippocampal slices for mGluRs studies

Male Wistar rats (Nossan strain, Milan), weighing 180–200 g, were used. After decapitation, their hippocampi were rapidly removed and placed in ice-cold Krebs-bicarbonate buffer: (mM) NaCl 122, KCl 3.1, $MgSO_4$ 1.2, KH_2PO_4 0.4, $CaCl_2$ 1.3, $NaHCO_3$ 25, and glucose 10. Transverse slices (350 µm thick) were cut from each hippocampus using a McIlwain tissue chopper and then left to stand, dipped into Krebs-bicarbonate solution gassed with 95% O_2/5% CO_2 for 1 h at 37 °C in order to allow functional recovery.

Measurements of adenosine 3′5′-cyclicmonophosphate (cyclic AMP) formation

Hippocampal slices (two per tube) were placed for 20 min in 500 µl of oxygenated (95% O_2, 5% CO_2) Krebs at 37 °C. Immediately following 5 µl of forskolin solution (final concentration, 30 µM) or vehicle (50% ethanol in water) and 5 µl of EAA agonist solution or its vehicle (water) were added in the presence of 1 mM of IBMX. Tubes were then placed in a shaking water bath for 15 min. Incubation was terminated by adding 0.75 ml of ice-cold 12 mM disodium EDTA solution to each tube. The samples were immediately homogenized using a Tetronix Tissuemixer and then boiled for 10 min. After centrifugation the supernatants were frozen at − 80 °C until cyclic AMP measurement. Cyclic AMP levels were determined using a cyclic AMP radioimmunoassay kit.

Studies on phosphatidylinositol hydrolysis

The slices, prepared as previously described, were incubated for 2 h with [^{3}H]inositol (20 µCi/ml). They were then washed in 50 ml of freshly oxygenated buffer and transferred to test-tubes (two slices each) with 500 µl of drug-containing medium and gently stirred in the presence of 10 mM LiCl by bubbling in 95% O_2/5% CO_2. After 15 min at 37 °C, the reaction was stopped by the addition of 1.88 ml of chloroform/methanol (1 : 2). The phases were separated by adding 0.65 ml of chloroform and 0.65 ml of water and, after brief sonication, by centrifuging the tubes at 800 × **g** for 10 min. The upper phase (2 ml) which contained the water-soluble [^{3}H] inositol phosphates (inositol monophosphate, IP; inositol 1,4-bisphosphate, IP_2; inositol 1,4,5-trisphosphate, IP_3) was transferred to test-tubes and water (3 ml) was added. The inositol phosphates were then separated on Dowex AG 1-X 8 anion exchange resin (formiate form, 100–200 mesh) as previously described (Ruggiero et al., 1987). The radioactivity in portions (8 ml) of these fractions was determined by liquid scintillation counting. Calculations were performed on the sum of IP, IP_2, and IP_3 (dpm/mg proteins).

Results

Effects of ammonium acetate on NMDA and AMPA receptors

The depolarization of cortical wedges induced by different concentrations of NMDA was reduced in a concentration-dependent manner by ammonium acetate (2–4 mM). Since sodium acetate did not modify the concentration-response curves of NMDA, this inhibition was ascribed to ammonium ions. Figure 1 shows that the effects of ammonium acetate were not competitive in nature. When a preparation was exposed for approximately 1 h to ammonium acetate and subsequently superfused with regular Krebs (for up to 90 min), the responses

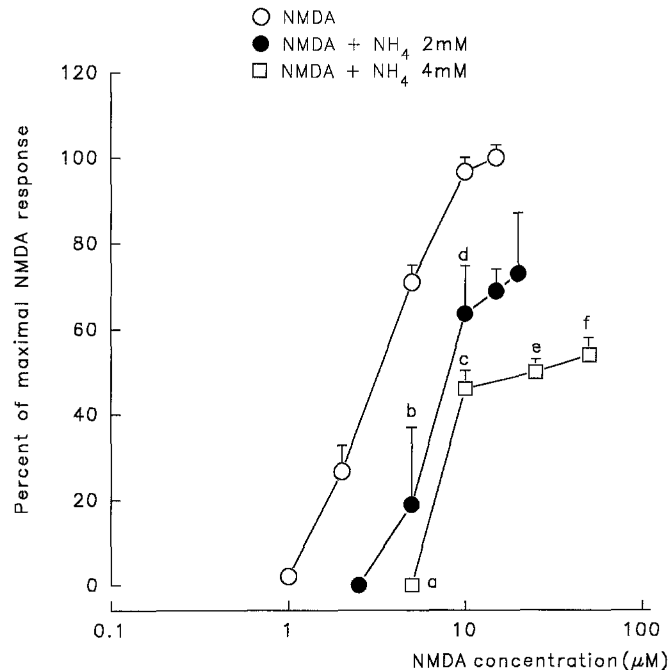

Fig. 1. The cortical wedge preparations were exposed to different concentrations of the agonist first in the absence and then in the presence of ammonium acetate (2–4 mM). In order to avoid the decrease in the degree of depolarization due to repeated and prolonged exposure of the preparations to the agonist, the points of the control curve tested, when the concentration response curve to ammonium acetate had to be done, were kept to a minimum (two or three). Each point represents the mean ± S.E. of at least 15 experiments for the control concentration-response curve and of at least 5 experiments for the curves obtained in the presence of ammonium acetate. The results were expressed as percent of the quasi-maximal NMDA effect (response obtained with 15 µM NMDA under stable experimental conditions before the treatment with ammonium acetate, see text for details). Statistical analysis was performed by analysis of variance and Tukey-Kramer test for multiple comparisons. a = $p < 0.001$ vs. NMDA 5 µM; b = $p < 0.01$ vs. NMDA 5 µM; c = $p < 0.01$ vs. NMDA 10 µM; d = $p < 0.05$ vs. NMDA 10 µM; e = $p < 0.01$ vs. NMDA 15 µM; f = $p < 0.05$ vs. NMDA 15 µM (maximal dose tested)

to NMDA remained significantly reduced. This suggests that in in vitro preparations it is difficult to reverse the actions of ammonium. However, the inhibitory effects of this ion did not increase by repeatedly challenging the preparation with a submaximal concentration of NMDA (10 µM), thus ruling out use-dependent antagonism of the NMDA receptor ion-channel function.

In a similar manner, toxic concentrations of ammonium acetate (2–4 mM) inhibited the responses of the cortical wedge preparations to AMPA. Figure 2 reports the effects of ammonium acetate on AMPA concentration-response curves, suggesting a non-competitive type of inhibition similar to that of NMDA. The inhibition of AMPA responses was not reversed by washing the slices with regular Krebs for 90 min, again suggesting that the actions of am-

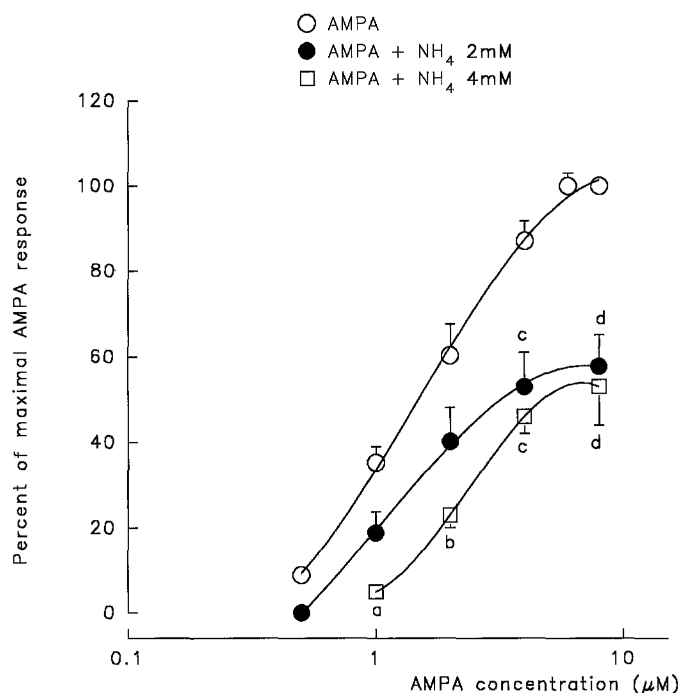

Fig. 2. The cortical wedge preparations were exposed to different concentrations of AMPA first in the absence and then in the presence of ammonium acetate (2–4 mM). Each point represents the mean ± S.E. of at least 12 experiments for the control concentration-response curve and of at least 5 experiments for the curves obtained in the presence of ammonium acetate. The results were expressed as percent of quasi maximal AMPA response (response obtained with AMPA 8 μM under stable experimental conditions, before the treatment with ammonium acetate; see text for details). Statistical analysis was performed by analysis of the variance and Tukey-Kramer test for multiple comparison. a = $p < 0.05$ vs. AMPA 1 μM; b = $p < 0.05$ vs. AMPA 2 μM; c = $p < 0.01$ vs. AMPA 4 μM; d = $p < 0.01$ vs. AMPA 8 μM

monium acetate on the ionotropic glutamate receptors do not easily reverse under in vitro conditions.

Effects of ammonium acetate on mGluRs

1S,3R-ACPD selectively stimulates most subtypes of mGluRs. In particular, by interacting with mGluR 1 and mGluR 5 it causes a significant increase in inositol phosphate accumulation and by acting on mGluR 2 and mGluR 4 it reduces the forskolin-induced accumulation of cyclic-AMP (Nakanishi, 1992). 1S,3R-ACPD acts also on other mGluR subtypes (mGluR 4, mGluR 6, and mGluR 7) but on these latter subtypes its potency is lower than that of L-AP 4 (Tanabe et al., 1993). We studied the interactions of ammonium acetate with 1S,3R-ACPD in rat hippocampal slices. Figure 3 shows that the effects of 1S,3R-ACPD on the accumulation of inositol phosphates were significantly reduced by toxic concentrations of ammonium ions. In a different series of experiments

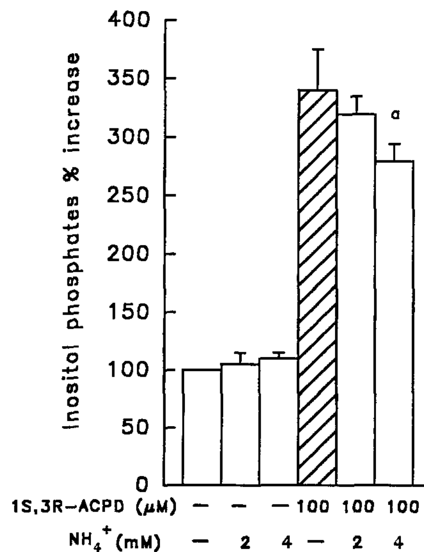

Fig. 3. Hippocampal slices prelabelled with [^{3}H]inositol and incubated in the presence of 10 mM LiCl were eventually challenged with 100 μM 1S,3R-ACPD for 15 min in the presence of ammonium acetate (2–4 mM). Data are expressed as percent increase over basal values in each experiment and are the means ± S.E. of at least 5 experiments run in triplicate. The basal value (100%) is the sum of radioactivity found in the fractions corresponding to [^{3}H]IP, [^{3}H]IP2, and [^{3}H]IP3 and was 23000 ± 2500 d.p.m/mg protein. [a]means $p < 0.05$ vs. 1S,3R-ACPD-induced increase of inositol phosphate formation; ANOVA and Dunnett's test

the effects of ammonium acetate were tested on the forskolin-induced (30 μM) accumulation of cyclic AMP. Figure 4 shows that ammonium directly inhibited forskolin effects. However, when ammonium and 1S,3R-ACPD (100 μM) were simultaneously present, the inhibition of forskolin-induced cyclic AMP accumulation was significantly increased and the effects of the two compounds were additive.

Discussion

Our experiments show that the responses evoked by the stimulation of both ionotropic and metabotropic glutamate receptors are profoundly affected by toxic concentrations of ammonium ions.

In particular, we showed that ammonium inhibits NMDA responses in a concentration-dependent manner. Previous studies on the interaction between ammonium and the NMDA receptor complex have shown that this ion reduces the number of NMDA sites in vivo and in vitro (Peterson et al., 1990; Rao et al., 1991) but does not affect [^{3}H]MK-801 binding (de-Knegt et al., 1993). The basic mechanism of ammonia-induced inhibition of NMDA function remains to be elucidated. We also showed that ammonium inhibits AMPA-induced depolarization in a concentration-dependent manner. It has previously

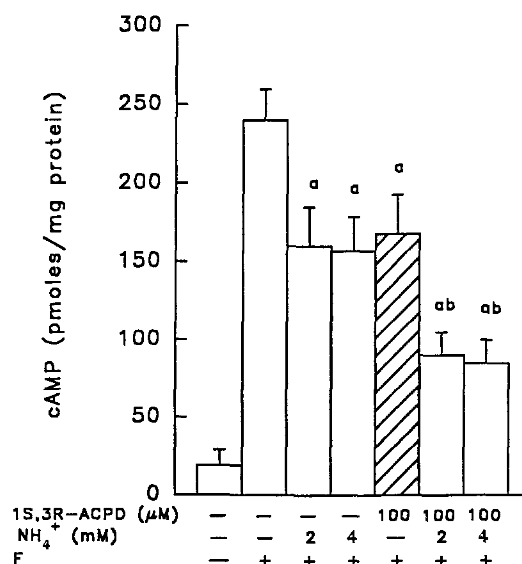

Fig. 4. Hippocampal slices eventually challenged with 30 μM forskolin in the presence of IBMX (for 15 min at 37 °C) were also treated with ammonium acetate (2–4 mM), with 1S,3R-ACPD or with the sum of both compounds. Each column represents the mean cyclic AMP content (pmoles/mg protein) present in the slices at the end of the experiment. Vertical bars are the means ± S.E. obtained in 5 experiments conducted in triplicate. [a]means $p < 0.01$ vs. forskolin treated slices. [b]means $p < 0.01$ vs. forskolin plus 1S,3R-ACPD treated slices

been reported that ammonia inhibits fast synaptic responses in hippocampal slices (Theoret et al., 1985), whose responses are mainly due to the interaction of synaptically released glutamate with AMPA receptors (Fagg et al., 1986). Our data are in line with these observations and further suggest that ammonium may directly modify the function of AMPA receptors. The effects of ammonium on both AMPA and NMDA receptors were not competitive in nature and were practically irreversible under in vitro conditions. This contrast with the observation that ammonia toxicity in vivo does not cause a permanent impairment of brain function. However, it has been recently described that elevated concentrations of ammonia significantly modify the function of different proteases and reduce the brain content of microtubule-associated proteins (MAP-2) (Felipo et al., 1993). These modifications of neuronal protein metabolism could explain why the actions of ammonium on ionotropic glutamate receptors in vitro were not reversible.

We also observed that ammonium inhibits the actions of 1S,3R-ACPD on the mGluRs which are linked to the stimulation of inositol phosphate formation, while functionally potentiating the effects of this agonist on the modulation of forskolin-induced cyclic AMP accumulation. Although the physiological role of mGluRs is not clear, one of the effects of the mGluR agonists is the presynaptic control of transmitter release (Herrero et al., 1992; Lombardi et al., 1993). Along this line, it has been shown that ammonium affects the depolar-

ization induced glutamate outflow both under in vitro and in vivo conditions (Hamberger et al., 1979; Moroni et al., 1983; Fan et al., 1990). The concept that both pre- and postsynaptic events of the glutamatergic synapses are affected by toxic concentrations of ammonia (Rao et al., 1992) completely agrees with the experimental observations here reported. A direct involvement of an abnormal stimulation of glutamate receptors in the pathogenesis of acute ammonia toxicity has also been proposed on the basis of a significant degree of protection exerted by glutamate receptor antagonists against the toxicity of ammonium acetate (Marcaida et al., 1992). This protection may occur because the antagonists further reduce the toxic effects of elevated concentrations of extracellular glutamate. In fact, beside the actions on glutamate receptor function described here, ammonia has been reported to increase glutamate levels in brain extracellular spaces (Moroni et al., 1983; Tossman et al., 1987) and to significantly affect the activity of enzymes directly related to glutamate metabolism. In particular, ammonium ions inhibit glutaminase (Kvamme and Lenda, 1982), aspartate amino transferase and alanine aminotransferase and enhance the activity of glutamine-synthetase and glutamate dehydrogenase (Rao et al., 1992 a, b). It is therefore not surprising that a profound derangement of the function of the excitatory glutamatergic synapses occurs when circulating concentrations of ammonia reach millimolar levels. These concentrations have been associated with signs and symptoms of profound neuronal dysfunction, leading to a comatose state or to death.

In conclusion, our results further support the concept that toxic concentrations of ammonia significantly change the function of the glutamatergic synapses. It is reasonable to assume that these changes are involved in the pathogenesis of the neurological events associated with increased concentrations of circulating ammonia.

Acknowledgements

Supported by National Research Council, the University of Florence and the European Community Biomed project N° PL 93-1033. We thank Dr. A. Cozzi for technical assistance.

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Received March 1, 1994