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Stimulation of [^{3}H]norepinephrine release from hippocampal slices by excitatory amino acids

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Abstract [^{3}H]norepinephrine efflux from preloaded rat hippocampal slices was increased in a dose-dependent manner by excitatory amino acids (EAA) in the following potency order: *N*-methyl-*D*-aspartic acid (NMDA) > kainic acid > *L*-glutamic acid ≥ *D,L*-homocysteic acid > *L*-aspartic acid > quinolinic acid > quisqualic acid. The effect of EAA was blocked by physiological concentration of Mg^{2+}, with the exception of kainic acid. *D,L*-2-amino-7-phosphonoheptanoic acid (APH) dose-dependently inhibited NMDA effect (IC_{50} = 69 μmol/L), whereas at 1 mmol/L it was ineffective versus kainic acid. The release of [^{3}H]norepinephrine induced by quinolinic acid was blocked by APH 0.1 mmol/L. γ-*D*-glutamylglycine dose-dependently inhibited kainic acid effect with an IC_{50} = 1.15 mmol/L. Tetrodotoxin 2 μmol/L reduced NMDA and kainic acid effects by 40 and 20%, respectively. The data indicate a possible involvement of central noradrenergic system in the modulation of excitotoxic action of EAA and offer a reliable system for testing new compounds acting at EAA-receptors by measuring norepinephrine release *in vitro*.

Key words [^{3}H]norepinephrine; excitatory amino acids; *D,L*-2-amino-7-phosphonoheptanoic acid; tetrodotoxin; magnesium; hippocampus

Glutamic acid, aspartic acid and their structurally related dicarboxylic amino acids have been shown to be able to excite and degenerate mammalian central neurons and to cause behavioral and electroencephalographic abnormalities highly reminiscent of human temporal lobe epilepsy[1]. Electrophysiological and binding studies of excitatory amino acids (EAA), named excitotoxins, led to the characterization of 3 distinct amino acid receptor subtypes preferring *N*-methyl-*D*-aspartic acid (NMDA), quisqualic acid (QA) and kainic acid, respectively [2]. Administration of convulsant EAA such as kainic acid, NMDA and quinolinic acid to rodents has been found to induce a depletion of brain norepinephrine (NE) associated with epileptoform activity[3]. Moreover, kainic acid-induced seizures and NE depletion could both be prevented by the pretreatment with anticonvulsants[4]. Thus, stimulation of NE out flow by EAA may be a functional response consequent to the paroxysmal activity of the neurons which underlies epileptic phenomena.

The present study was undertaken to investigate the effects of various EAA and their receptor subtype antagonists on [^{3}H] NE release in rat hippocampal slices. The role of Mg^{2+} on the NE release induced by EAA was also evaluated.

Materials and methods

Biochemical assay Sprague-Dawley ♂ rats (237 ± SD 23 g) were decapitated and the hippocampus was rapidly dissected on ice. Experimental conditions for [^{3}H]NE release from brain slices were chosen according to the method of NE determination *in vitro*[4,5] with some modifications. Hippocampal pairs were mechanically chopped in the transversal plane at a thickness of 300 μm. The slices were pooled and suspended in 5 ml oxygenated Krebs-bicarbonate buffer (OKB), pH 7.6, containing ascorbic

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acid (100 mg/L) and pargyline (50 mg/L). After 15 min preincubation at 37℃, [^{3}H] NE 0.1 μmol/L (740 GBq/mmol, radiochemical purity more than 90%, NEN, Boston) was added and the slices were incubated for another 20 min. The supernatant fluid was decanted off and the slices were washed 3 times with fresh OKB. Each washing was followed by 5 min incubation. The slices (approximately 30 mg wet weight per tube) were transferred to the tube containing 500 μl OKB and incubated at 37℃. An aliquot of 250 μl was withdrawn every 5 min from each tube and an equal amount of fresh OKB was added. Baseline release was determined after a 30-min washout period. Drugs were diluted in OKB and added in 250 μl after 0.5 h of baseline release. EAA were added to the slices in OKB for 10 min, EAA antagonists were added 5 min before the agonists and in the presence of the agonists for the next 10 min. After drug addition a 30 min washout period with drug-free OKB was studied. The drugs were applied at the concentration as shown in the tables and figures.

The radioactivity content was assessed in the various fractions by scintillation spectrometry. Slices were recovered at the end of the experiment and dissolved in 1 ml soluene (Packard Dawners Grove, ILL). Tritium content was determined in same way. 90% of the radioactivity was recovered as [^{3}H]NE, indicating the lack of significant metabolism of the [^{3}H] catecholamine during the experi-

Fractional release was calculated as

ment.

tritium released into the medium during each 5 min interval as a percentage of the tritium content of the slices during that interval. The effect of the drugs was calculated as S_2/S_1: the fractional release during drug application (S_2) above the baseline of spontaneous release (S_1). Spontaneous release was assessed as the average of three samples before drug application.

The relative potencies of the various EAA in inducing [^{3}H]NE release were calculated from the dose at which each agonist caused a 100% increase in [^{3}H]NE outflow over baseline. This dose was extrapolated for each drug from the corresponding dose-response regression-line. The IC_{50} for the antagonists (concentration which inhibited the maximal effect of a given agonist by 50%) was similarly calculated on the regression line for the inhibition induced by various doses of the antagonist of the effect of a given agonist dose.

Materials: EAA and tetrodotoxin (TTX) were purchased from Sigma Co. EAA antagonists were obtained from Cambridge Research Biochem. EAA-agonists and antagonists were dissolved in NaOH 1 mol/L. The solutions were adjusted to pH 7.4 with HCl 1 mol/L and diluted to the final concentration with OKB.

Statistical analysis *t*-Test and Dunnett's test (two-tailed comparison) were used for single and multiple comparisons, respectively.

Results

After 1 h perfusion, spontaneous [^{3}H] NE release was stabilized at a constant rate of about 1% of the total [^{3}H] in the slices per min. This rate was mantained for an additional hour, so our experiments were concluded within 120 min. [^{3}H]NE release was stimulated as expected by KCl 50 mmol/L to 5 times over baseline in a Ca^{2+}-dependent manner (data not shown). These results were in accordance with previous findings in similar preparations[4,8].

Preliminary experiments showed that [^{3}H]NE releasing activity of EAA, with the exception of kainic acid, was inhibited by the presence of Mg^{2+}. Thus, in all subsequent experiments Mg^{2+} was omitted from the incubation medium. This did not significantly affect spontaneous [^{3}H]NE release.

Tab 1 shows the Mg^{2+} dependence of [^{3}H] NE release induced by various EAA

tested. The ability of all the compounds to release [^{3}H] NE were inhibited in the presence of $MgCl_2$ 1.2 mmol/L when compared to a Mg^{2+} free solution, with the exception of kainic acid. At $MgCl_2$ 0.15 mmol/L, a concentration 8 times lower than the physiological value, NMDA (0.1 mmol/L)-induced [^{3}H]NE release (S_2/S_1 = 1.34±0.05) was inhibited by 45%; at $MgCl_2$ 0.6 mmol/L the effect of NMDA was abolished.

Tab 1. Mg^{2+} dependence of the release of [^{3}H]NE caused by excitatory amino acids. n≥4 determinations, $\bar{x}\pm SD$. ***$P<0.01$ *vs* -$MgCl_2$ group.

Drugs	mmol/L	Fractional release (S_2/S_1) -$MgCl_2$	+$MgCl_2$(1.2 mmol/L)
NMDA	0.1	2.45±0.27	0.96±0.13***
Quin	1	1.82±0.29	0.95±0.12***
D,L-Hc	2	3.52±0.91	1.07±0.04***
L-Glu	1	2.12±0.36	1.19±0.19***
L-Asp	2	2.69±0.27	0.95±0.11***
KA	0.25	3.37±0.41	3.30±0.26

NMDA: *N*-methyl-*D*-aspartic acid. Quin: quinolinic acid. *D,L*-Hc: *D,L*-homocysteic acid. *L*-Glu: glutamic acid. *L*-Asp: *L*-aspartic acid. KA: kainic acid.

The relative potencies in inducing [^{3}H] NE release of various EAA acting at distinct receptor sites were evaluated. All compounds were tested at least at 3 concentrations between the minimal and the maximal effect in releasing [^{3}H]NE. The effect of the EAA was reversible, declining when they were removed from the incubaion medium. The relative potency of EAA as estimated by the concentration eliciting a release of [^{3}H] NE twice the baseline value, took the following rank order: NMDA (60 μmol/L)>kainic acid (125 μmol/L)>*L*-glutamic acid (340 μmol/L)≥*D,L*-homocysteic acid (380 μmol/L)>*L*-aspartic acid (832 μmol/L)>quinolinic acid (925 μmol/L). QA was inactive. To investigate whether the [^{3}H] NE release induced by various EAA was receptor-specific, we tested the inhibition of such effect by several EAA antagonists: *D,L*-2-amino-7-phosphonoheptanoic acid (APH), *D,L*-2-amino-5-phosphonovaleric acid (APV) and γ-*D*-glutamylglycine (γ-DGG).

The effects of APH, a selective NMDA receptor blocker[7], on the NMDA agonist- and kainic acid-induced [^{3}H]NE release are shown in Tab 2. The efflux induced by quinolinic acid, a preferential agonist at NMDA receptors[8], at a concentration of 1 mmol/L was completely blocked by APH 0.1 mmol/L. APH caused a dose-dependent inhibition of [^{3}H] NE release induced by NMDA 0.1 mmol/L, with an IC_{50} of 0.069 mmol/L (95% confidence limits (CL) = 55 - 90); complete antagonism was obtained with APH 0.2 mmol/L. In contrast, the effect of kainic acid 0.25 mmol/L was unchanged in the presesence of APH 1 mmol/L. APV, another established NMDA receptor blocker[5], at 0.1 mmol/L also suppressed the quinolinic acid effect (data not shown).

Tab 2. Effects of *D,L*-2-amino-7-phosphono-heptanoic acid (APH) on excitatory amino acid receptor agonist-induced release of [^{3}H]NE. n≥4 determinations, $\bar{x}\pm SD$. ***$P<0.01$ *vs* APH-free group.

APH (mmol/L)	Fractional release (S_2/S_1) NMDA 0.1	Quin 1	KA 0.25 mmol/L
0	2.48±0.30	1.82±0.29	3.37±0.41
0.1	1.15±0.21***	1.03±0.18***	3.44±0.43
1.0	1.02±0.41***	1.04±0.26***	3.67±0.39

As shown in Fig 1, γ-DGG, an antagonist at both NMDA and kainic acid receptors, dose-dependently reduced the release induced by kainic acid 0.25 mmol/L, with an IC_{50} of 1.15 mmol/L (95% CL = 0.8 - 1.4). A maximal inhibition of 62% was reached at γ-DGG. 2 mmol/L In the concentration range tested none of the antagonists significantly modified the spontaneous efflux of [^{3}H]NE.

Fig 1. Dose-dependent antagonism by γ-*D*-glutamylglycine of the kainic acid (0.25 mmol/L)-induced release of [^{3}H]NE. At least 4 determinations per dose. $\bar{x} \pm$SD. $^{***}P<0.01$, Dunnett's test *vs* kainic acid group.

To investigate whether the effect of EAA on the release of [^{3}H]NE involved generation of action potentials, we evaluated the sensitivity of such effects to tetrodotoxin (TTX), a Na^+ channel blocker. At 2 μmol/L, a dose which did not modify the spontaneous [^{3}H]NE release, TTX partially blocked the action of NMDA 0.1 mmol/L and kainic acid 0.25 mmol/L as shown by 40 and 20% inhibition, respectively of their effects (NMDA: $S_2/S_1 = 2.52 \pm 0.24$; NMDA+TTX: $S_2/S_1 = 1.43 \pm 0.06$; kainic acid: $S_2/S_1 = 3.15 \pm 0.22$; kainic acid +TTX: $S_2/S_1 = 2.55 \pm 0.26$, n=6).

NMDA and kainic acid effects were significantly reduced when Ca^{2+} was omitted and EGTA added in OKB (data not shown).

Discussion

In the present study, various EAA were found to stimulate the spontaneous release of [^{3}H]NE. In hippocampal slices the afferent fibers are separated from their cell bodies but the local network is still efficient. Thus, [^{3}H]NE release may be locally regulated by EAA. TTX partially inhibited both kainic acid and NMDA action, supporting previous evidence[4] that trans-synaptic mechanisms are involved in [^{3}H]NE release caused by EAA.

A rank order of potency to release NE by EAA was found similar to that obtained in depolarization and Na^+ efflux studies in hippocampal slices[9]: NMDA and kainic acid were the most powerful while *L*-aspartic acid and quinolinic acid were the least effective. QA did not stimulate [^{3}H]NE release in contrast with its ability to depolarize hippocampal neurons with a potency similar to NMDA[10] suggesting that the mechanisms such as uptake or enzymatic inactivation which rapidly removes QA from the extracellular space[11] may play a role.

The striking sensitivity of NMDA, but not kainic acid effect, to very low concentrations of Mg^{2+} may mean that Mg^{2+} selectively impairs NMDA receptor function [12]. Extracellular physiological concentration of Mg^{2+} plays a critical inhibitory role in the "switching on" the voltage-dependent NMDA channel which may be closely linked to the neurotransmitter release. Similarly, the effects of putative ligands such as *D,L*-homocysteic acid, quinolinic acid, *L*-aspartic acid and glutamic acid, at NMDA receptors are inhibited by Mg^{2+}. Although classified as "mixed" agonists[1], *L*-aspartic acid and *L*-glutamic acid seemed to activate preferentially NMDA receptors to induce NE release.

APH blocked the NMDA effect at doses shown to antagonize the depolarizing properties of the EAA in various preparations[8]; similarly, APH abolished the effect of quinolinic acid. Kainic acid effect was unchanged by APH but blocked by γ-DGG, a "mixed" EAA antagonist.

The present study has shown that [^{3}H]NE release can be stimulated by a broad spectrum of EAA in rat hippocampal slices. This represents an advantage over other biochemical methods aimed at coupling a functional response to the effector interaction with its receptor[13,14].

For instance, while Na^+ fluxes across the neuronal membrane[15] represents an event more directly coupled to the agonist-receptor interaction but the effect rapidly decays after the application of the agent and the full dose-response curve can not be determined. The biochemical measurement described in this study offers a reliable system for testing new compounds with potential antagonist activity at EAA-receptors.

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兴奋性氨基酸促进大鼠海马切片[^{3}H]去甲肾上腺素的释放

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提要 兴奋性氨基酸能增加大鼠海马切片[^{3}H]去甲肾上腺素(NE)的释放，强度次序为*N*-甲基-*D*-门冬氨酸(NMDA)＞卡因酸＞*L*-谷氨酸≥*D*, *L*-高半胱氨酸＞*L*-门冬氨酸＞喹啉酸＞使君子氨酸。该促释放作用除卡因酸外均为生理浓度 Mg^{2+} 所拮抗。*D*, *L*-2-氨基-7-膦酸基庚酸(APH)拮抗NMDA和喹啉酸但不影响卡因酸的促NE释放。γ-D-谷酰甘氨酸消除卡因酸的促NE释放。河豚毒素使NMDA和卡因酸的促释放作用分别下降40和20%。结果提示中枢去甲肾上腺素能系统参与兴奋性氨基酸的兴奋毒作用。

关键词 [^{3}H]去甲肾上腺素；兴奋性氨基酸；*D*,*L*-2-氨基-7-膦酸基庚酸；河豚毒素；镁；海马