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N-Acetylaspartylglutamate: An endogenous peptide with high affinity for a brain "glutamate" receptor

(neuropeptides/neurotransmitters/neuroexcitant)

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ABSTRACT **A brain peptide with high affinity (420 nM) and marked specificity for brain receptor sites labeled with L-[^{3}H]glutamate has been identified. Amino acid analysis and mass spectroscopy indicate that the peptide is *N*-acetylaspartylglutamate. The peptide exhibits potent convulsant properties when injected into the rat hippocampus, similar to those produced by the glutamate receptor agonist, quisqualic acid. These findings raise the question whether endogenous brain peptides enriched in acidic amino acids may serve as excitatory neurotransmitters.**

The acidic amino acids L-glutamate and L-aspartate have potent excitatory effects when iontophoresed onto brain neurons (1–3). A binding site for L-[^{3}H]glutamate has been described in the mammalian brain, which, on the basis of its subcellular distribution, pharmacologic characteristics, and ontogeny, appears to represent an excitatory receptor responsive to L-glutamate (4–7). Considerable evidence suggests that L-glutamate or L-aspartate, or both, may serve as excitatory neurotransmitters in the mammalian brain (1–3). However, recently reported disparities in the ability of antagonists to block the excitatory effects of exogenously applied glutamate or aspartate, as compared to the endogenous neurotransmitter released by putative glutamatergic neurons, have raised questions about this conclusion (8, 9).

In this report, we demonstrate that *N*-acetylaspartylglutamate (Ac-Asp-Glu), an endogenous brain peptide, exhibits a high affinity and specificity for brain receptor sites labeled with L-[^{3}H]glutamate. Intrahippocampal injection of Ac-Asp-Glu caused a prolonged seizure disorder resembling that observed after infusion of quisqualate, a selective excitatory receptor agonist; in contrast, higher doses of L-glutamate were devoid of convulsive effects. These findings raise the question whether endogenous peptides containing acidic amino acids may be involved in excitatory neurotransmission.

METHODS

Peptide Purification. Immediately after decapitation, whole rat brains were removed and rapidly homogenized in 20 vol of ice-cold 0.4 M perchloric acid; the homogenate was centrifuged at 10,000 × *g* for 10 min at 5°C. The supernatant fluid was neutralized with 5.0 M KOH, and the $KClO_4$ precipitate was removed by filtration. The filtrate was applied to a Dowex AG-50X8 column to remove primary amines, including endogenous glutamate and aspartate. The column was washed with 100 ml of distilled water, and the total effluent was collected. After titration to pH 6.5 with KOH, this fraction was applied to a Dowex AG-1X8 column and was eluted with a linear gradient consisting of distilled water and 4 M formic acid. Active fractions (2.25 M formate) were combined, lyophilized, and dissolved in 0.1 M potassium phosphate buffer (pH 5.0) and were purified further by HPLC on a 10-μm Vydac SC-anion exchange column (25 cm × 0.45 cm inside diameter) with absorbance monitored at 215 nm.

Peptide Characterization. The fractions were lyophilized, reconstituted in 50 mM Tris·HCl (pH 7.1), and assayed for their ability to inhibit the specific binding of L-[^{3}H]glutamate to rat brain membranes (4, 6). Extensively washed membranes prepared from adult rat midbrain or cerebral cortex (100 μg of protein/ml) were incubated for 20 min at 37°C in a volume of 1 ml of 50 mM Tris·HCl buffer containing 100 nM L-[^{3}H]glutamic acid (32 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq; New England Nuclear) and the dissolved chromatographic fractions. Nonspecific binding was determined in the presence of 500 μmol of unlabeled L-glutamate. The receptor–ligand complex was isolated by rapid centrifugation at 10,000 × *g* at 5°C. The pellet was washed superficially with glass-distilled water and solubilized in Protosol (New England Nuclear); radioactivity was measured by scintillation spectrometry. In addition, the possible effects of active fractions on the specific binding of [^{3}H]muscimol (1.8 nM; New England Nuclear) to cerebellar γ-aminobutyric acid receptors (10), [^{3}H]kainic acid (25 nM; Amersham/Searle) to striatal kainate receptors (11), L-[^{3}H]quinuclidinyl benzilate (0.3 nM; New England Nuclear) to cortical muscarinic receptors (12), and [^{3}H]spipirone (1 nM; New England Nuclear) to striatal dopamine receptors (13) were also examined.

The amino acid content of native fractions and fractions hydrolyzed in 6 M HCl at 100°C for 12 hr was determined by the precolumn derivatization technique of Hill *et al.* (14). In addition, the HPLC-purified compound was subjected to characterization by mass spectroscopy (MS) on a Kratos MS-50 instrument equipped with a DS-55 data system. The purified compound was derivatized by permethylation and by trimethylsilylation.

Electroencephalography. After anesthesia with pentobarbital-chlorohydrate, unipolar electrodes were inserted into the cerebral cortex at the pial surface and a reference electrode was inserted into the olfactory bulb. The electrodes were affixed with dental cement. Seven days after surgery, the electrodes were connected with a Grass 6-channel polygraph with electroencephalogram (EEG) preamplifier, and 1 μl of drug or purified Ac-Asp-Glu, dissolved in isotonic saline, was injected via a 0.3-mm Hamilton cannula into the hippocampal formation over a period of 1 min during ether anesthesia (15). The EEG was recorded in the unrestrained rat over the next 2 hr.

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Abbreviations: Ac-Asp-Glu, *N*-acetylaspartylglutamate; EEG, electroencephalogram; MS, mass spectroscopy.

RESULTS

Purification of the Peptide with L-[^{3}H]Glutamate Displacement Activity. Although fractions from many regions of the gradient from the Dowex AG-1 column inhibited the specific binding of L-[^{3}H]glutamate, a peak of displacement activity at fractions centering around 2.25 M formic acid showed the greatest potency upon serial dilution. No free glutamate or aspartate was detected in this portion of the gradient (<0.1 pmol/ml); however, hydrolysis of these active fractions yielded a peak of glutamate that was isographic with the fractions exhibiting the greatest displacement potency. These active fractions were combined, lyophilized, and then dissolved in 0.1 M potassium phosphate buffer (pH 5.0); they were purified further by HPLC with absorbance monitored at 215 nm (16). Aliquots of the fractions isolated from the HPLC gradient were assayed for displacement activity at the L-[^{3}H]glutamate binding site and also were subjected to acid hydrolysis for measurement of the endogenous amino acids. As shown in Fig. 1, the peak of L-[^{3}H]glutamate displacement activity eluted primarily with one of the peaks detected by UV absorbance. Acid hydrolysis of the HPLC eluent fractions indicated that the peak of L-[^{3}H]glutamate displacement activity (fractions 9–12) contained equimolar concentrations of glutamate and aspartate. Notably, the preceding peak of UV absorbance (fractions 4–8), which contains primarily aspartate on hydrolysis, was inactive in the binding assay. This earlier peak cochromatographs with *N*-acetyl aspartate, which does not significantly inhibit the binding of L-[^{3}H]glutamate at 0.1 mM.

The HPLC-purified compound was subjected to further characterization by MS. Fast atom-bombardment MS indicated a molecular weight of 304 and secondary molecular peaks, suggesting the existence of three anionic sites consistent with carboxyl groups. Electron-impact MS of the permethyl derivative revealed fragment ions, indicating that an *N*-acetyl group was attached to the aspartyl residue (17, 18). Fragment ions consistent with an Asp-Glu sequence also were observed. High-resolution electron-impact MS of the trimethylsilyl derivative confirmed the *N*-acetylaspartyl group. Thus, based upon molecular weight, the amino acid content, and MS fragmentation characteristics, the compound of interest appears to be Ac-Asp-Glu.

Ligand–Receptor Interactions of Ac-Asp-Glu. Ac-Asp-Glu exhibits both a high affinity and a high degree of specificity for the receptor site labeled with L-[^{3}H]glutamate. Fig. 2 demonstrates that Ac-Asp-Glu, purified through the HPLC step, competes at a subset of sites in rat cortex membrane preparations specifically labeled with L-[^{3}H]glutamate. The Eadie–Hofstee plot of Ac-Asp-Glu displacement reveals a mass-action type of kinetics of inhibition with an apparent K_i of 0.42 μM and a maximal displacement of 40% of the specifically bound L-[^{3}H]glutamate. In contrast, the K_i of L-glutamate itself is 1.8 μM, and a K_i of quisqualic acid, the most potent conformationally restricted excitatory analogue at this receptor, is 0.6 μM (7). In membranes prepared from the rat midbrain, Ac-Asp-Glu produced a maximal displacement of L-[^{3}H]glutamate of 55% with an apparent K_i of 0.44 μM. Thus, Ac-Asp-Glu exhibits the highest affinity for the brain sites labeled with L-[^{3}H]glutamate of any compound thus far examined.

To determine the specificity of the interactions of Ac-Asp-Glu with the subpopulation of glutamate receptors, the effects of 10 μM Ac-Asp-Glu, which produced maximal displacement at the L-[^{3}H]glutamate-labeled site, on several other receptor sites were examined (Table 1). This concentration of Ac-Asp-Glu did not inhibit significantly the specific binding of [^{3}H]kainic acid to striatal kainate receptors, [^{3}H]muscimol to cerebellar γ-aminobutyric acid receptors, [^{3}H]quinuclidinyl benzilate to cortical muscarinic receptors, or [^{3}H]spiroperidol to striatal dopamine 2 (D2) receptors. Thus, the potent inhibitory effects of Ac-Asp-Glu at receptor sites appear to be limited to the subset of excitatory receptors labeled with L-[^{3}H]glutamate.

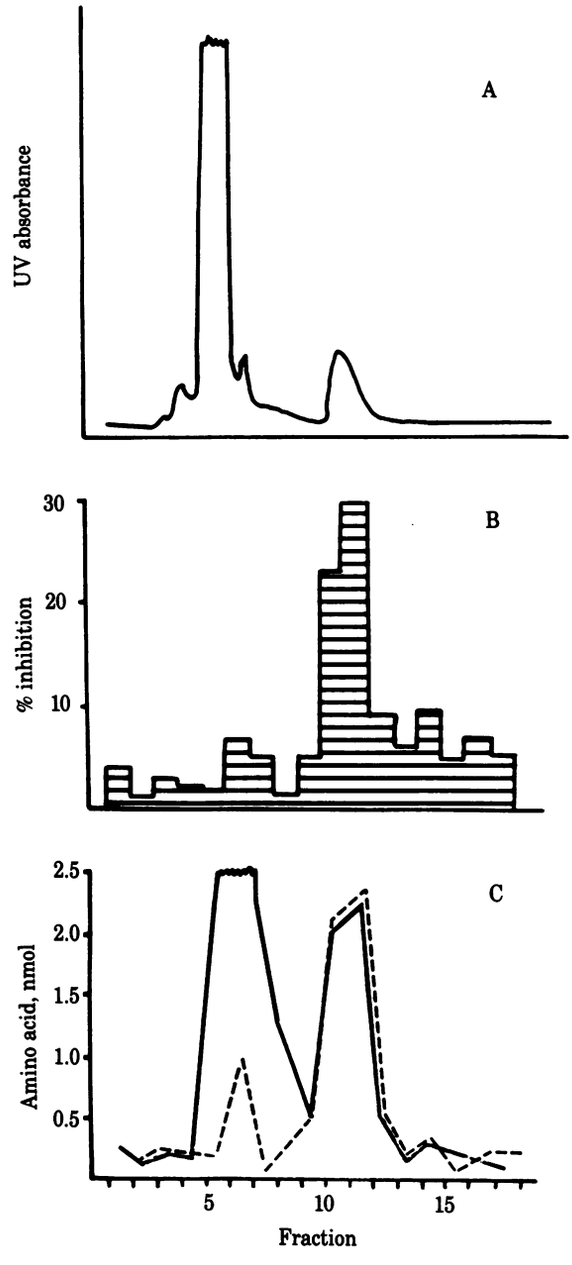

FIG. 1. HPLC separation of the active component. The peak of L-[^{3}H]glutamate displacement activity in the 2.25 M formate fraction of the Dowex AG-1 column was subjected to further purification by HPLC over a 10-μm Vydac SC-anion exchange column. A gradient of 0.25 M KCl (0–50%), with 0.1 M potassium phosphate buffer (pH 5.0) as the mobile phase, was used to elute the compound. (*A*) UV absorbance at 215 nm. (*B*) Ability of the fractions to inhibit the specific binding of L-[^{3}H]glutamate (100 nM) to washed membranes prepared from the rat cerebral cortex. (*C*) Amino acid content (——, Asp and – – –, Glu) of the fractions after acid hydrolysis as determined by the HPLC precolumn derivatization technique of Hill *et al.* (14).

To rule out the possibility that Ac-Asp-Glu displacement of L-[^{3}H]glutamate resulted simply from the L-glutamate liberated from the peptide, two experiments were performed. First, after incubation of the membranes in the L-[^{3}H]glutamate binding assay, the concentration of free glutamate in the supernatant

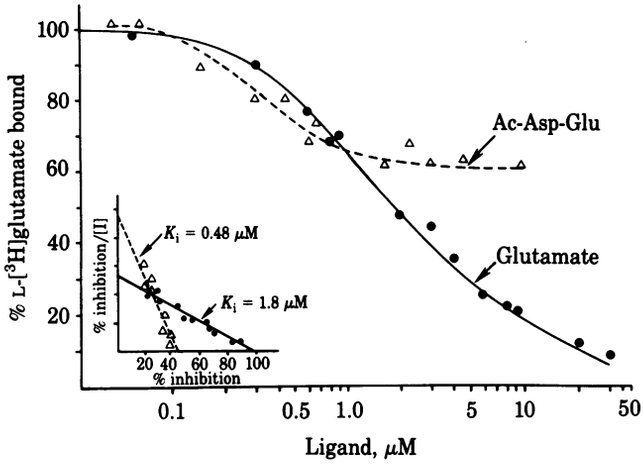

FIG. 2. Kinetics of inhibition of L-[^{3}H]glutamate binding by purified Ac-Asp-Glu. Washed membranes prepared from rat cerebral cortex were incubated with L-[^{3}H]glutamate (100 nM) (●) and various concentrations of HPLC-purified Ac-Asp-Glu (△) or unlabeled L-glutamate. L-Glutamate exhibits a mass-action type of kinetics of inhibition of the specific binding of L-[^{3}H]glutamate with complete displacement at 10 μM. In contrast, Ac-Asp-Glu exhibits a maximal displacement of 40% of the specifically bound L-[^{3}H]glutamate at 10 μM. Eadie–Hofstee plots for glutamate revealed a maximal binding (B_{max}) of 19.5 pmol/mg of protein with a K_i of 1.8 μM and for Ac-Asp-Glu, a B_{max} of 8.7 pmol/mg of protein with a K_i of 0.48 μM. [I], inhibitor concentration.

was determined in preparations to which Ac-Asp-Glu (10 μM) was or was not added. The content (±SEM) of free glutamate in the supernatant from membranes incubated with Ac-Asp-Glu (125 ± 17%) did not differ significantly from control membranes in the absence of Ac-Asp-Glu ($P > 0.1$; $n = 8$). Secondly, Ac-Asp-Glu was subjected to acid hydrolysis, and the neutralized hydrolysate was examined for displacement activity at the L-[^{3}H]glutamate binding site. The hydrolysate was less potent than Ac-Asp-Glu and exhibited a displacement curve virtually identical to authentic L-glutamate (e.g., complete displacement).

Convulsant Effects of Ac-Asp-Glu. Because intrahippocampal injection of excitatory analogues of glutamate causes characteristic seizure disorders (15), we examined the neurophysiological effects of Ac-Asp-Glu in rats monitored by cortical electroencephalography. After stereotaxic infusion of 125 nmol of Ac-Asp-Glu into the dentate gyrus, the rats developed a seizure disorder that evolved over several hours and resembled that following the infusion of quisqualic acid. By 20 min after Ac-Asp-Glu infusion, intermittent spikes appeared on the EEG, and the first sustained seizure occurred 20 min later (Fig. 3).

Table 1. Effects of Ac-Asp-Glu on receptor binding

Ligand			
Compound	Concentration, nM	Region	% inhibition
L-[^{3}H]Glutamate	100	Midbrain	55 ± 5
[^{3}H]Kainic acid	25	Striatum	1 ± 3
[^{3}H]Muscimol	1.8	Cerebellum	5 ± 5
[^{3}H]Spipirone	1	Striatum	1 ± 1
[^{3}H]Quinuclidinyl benzilate	0.3	Cortex	−6 ± 2

Specific binding of the ligands to washed membrane preparations was assessed in the regions indicated in the presence and absence of 10 μM of HPLC-purified Ac-Asp-Glu. The results are shown as the mean ± SEM of the % inhibition of specific binding measured in five or more separate preparations assayed in duplicate.

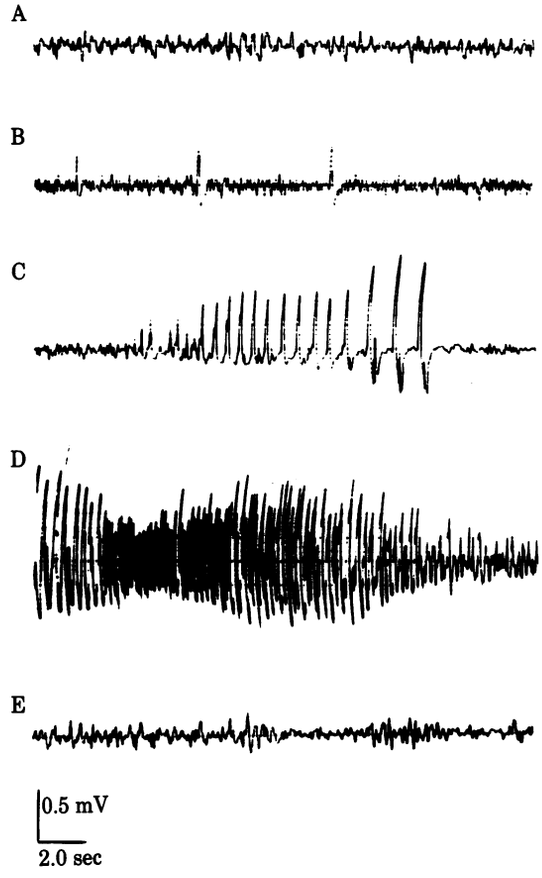

FIG. 3. Electroencephalographic recordings after intrahippocampal injection of Ac-Asp-Glu. Unipolar electrodes were inserted at the level of the pial surface of the cortex and were affixed to connectors on the calvarium. Ten days after surgery recordings were obtained in a shielded and grounded cage with a Grass 6-channel polygraph with an EEG preamplifier. The recordings commenced 3–5 min after intrahippocampal infusion of drugs in ether-anesthetized rats. Recordings shown were obtained from the parietal cortex contralateral to the side of the injection. The vertical bar = 0.5 mV and the horizontal bar = 2 sec. (*A*) Control pattern prior to injection. (*B*) Thirty-five minutes after injection of Ac-Asp-Glu; note the repetitive spiking. (*C*) Seizure episode 68 min after injection of Ac-Asp-Glu. (*D*) Seizure episode 135 min after injection of Ac-Asp-Glu associated with motor manifestations. (*E*) EEG 60 min after injection of 250 nmol of L-glutamate; note absence of seizure activity.

The seizure episodes became repetitive by 1 hr after infusion and reached their peak intensity at 2 hr. During the seizures, the rats exhibited forelimb hyperextension, clonus, retropulsion, rearing, and head bobbing; less intense EEG seizures were associated with immobility and staring. Threshold effects of Ac-Asp-Glu associated with cortical spiking were observed with doses as low as 25 nmol. The dosage range, behavioral abnormalities, and EEG seizure patterns associated with Ac-Asp-Glu injection were similar to those resulting from injection of comparable concentrations of quisqualic acid. To preclude the possibility that the seizures resulted from the liberation of glutamate from Ac-Asp-Glu, 250 nmol of L-glutamate (in 1 μl) were infused into the dentate gyrus. In contrast to the effects of Ac-Asp-Glu, L-glutamate caused only minimal and transient EEG abnormalities limited to the first 15 min after injection, and no behavioral alterations were observed.

DISCUSSION

These studies indicate that the endogenous peptide Ac-Asp-Glu exhibits high affinity and considerable specificity for a subpopu-

lation of brain receptor sites labeled with L-[^{3}H]glutamate. The percentage of maximal displacement of L-[^{3}H]glutamate varies among different brain regions; thus, it appears that Ac-Asp-Glu interacts with only a portion of the receptors that bind L-[^{3}H]glutamate. Notably, Ac-Asp-Glu does not displace [^{3}H]kainate, which is thought to act at a population of excitatory receptors distinct from those responsive to ionophoretically applied L-glutamate, quisqualate, or *N*-methyl-D-aspartate (3). Furthermore, Ac-Asp-Glu exhibits convulsant properties similar to those previously described for quisqualic acid, the next most potent displacer at the receptor sites labeled with L-[^{3}H]glutamate (6, 15).

Previous studies have shown that Ac-Asp-Glu is found in high concentrations (e.g., 0.5 μmol/g) in the brain but not in peripheral tissues (19, 20). Furthermore, Ac-Asp-Glu has an uneven distribution in the mammalian brain, with the greatest concentration in the midbrain and brainstem (16, 20, 21). Though the physiological role played by Ac-Asp-Glu remains obscure, it is reported to have excitatory effects when ionophoretically applied on cortical neurons (22). Recently, Kanazawa and Sutoo (23) have reported that transection of the spinal cord results in a selective depletion of an NH_2-blocked peptide containing aspartate and glutamate in the spinal cord distal to the lesion. This compound, which has been tentatively identified as hydroxybutyrylaspartylaspartylglutamate, has been found also to have excitatory effects when ionophoretically applied to spinal neurons (24). These findings raise the question whether NH_2-blocked peptides enriched in acidic amino acids might serve as endogenous excitatory neurotransmitters. In this regard, it is intriguing to note that the sodium-dependent, high-affinity synaptosomal uptake process for L-glutamate also transports aspartate, thereby concentrating these two amino acid precursors for Ac-Asp-Glu and hydroxybutyrylaspartylaspartylglutamate in excitatory terminals (25).

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