A Peptide Containing Aspartic Acid, Glutamic Acid and Serine in Calf Brain Synaptic Vesicles

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Free amino acids and other amino compounds in calf brain synaptic vesicles were identified and determined by thin-layer chromatography and ion-exchange chromatography. The vesicles contained ten identified amino acids with glutamic acid, aspartic acid, taurine and γ -aminobutyric acid in the highest concentrations, and also cysteic acid (or cysteinesulfinic acid), glutamine, alanine, serine, glycine and lysine. The vesicles also contained certain unknown acid-labile, ninhydrin-positive compounds, one of which was a peptide yielding, after acid hydrolysis, about 40 % aspartic acid, 30 % serine, 15 % glutamic acid, 10 % glycine and possibly some alanine and lysine. The concentration of the peptide in the vesicles was as high as that of all the other amino compounds together.

The vesicles of nerve terminals store transmitter substances, among which acetylcholine and catecholamines are well-known. In addition, eight to nine amino acids have been reported in the vesicles of mammalian cerebral cortex.¹⁻³ Several peptides have been found in the mammalian central nervous system⁴ and Reichelt and Kvamme⁵ reported *N*-acetyl-aspartyl-peptides containing various transmitter candidates in the central nervous system, but no one has yet reported these in the vesicles. When analysing free amino acids in calf brain synaptic vesicles we found a low-molecular weight peptide yielding mainly aspartic acid, glutamic acid and serine after acid hydrolysis. This observation is reported in preliminary form elsewhere.⁶ Here a complete analysis is given of the amino compounds in calf brain synaptic vesicles.

EXPERIMENTAL

The vesicles were prepared from newly-removed calf brain cortical layer according to the sucrose gradient centrifugation method of Morgan *et al.*⁷ In addition to this normal sucrose gradient, we also used a denser gradient containing equal volumes of 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 1.0 and 1.2 M sucrose solutions (Fig. 1). When checked under an electron microscope, the isolated vesicles were found to be of good quality and high purity. The yield of the vesicles was about 0.18 mg vesicle protein per 1 g wet weight of the cortex. Data concerning their homogeneity are presented elsewhere.⁶ Soluble cytoplasmic impurities were washed off with 2 × 5 ml of 1 mM potassium phosphate buffer (pH 7.5) containing 0.1 mM ethylenediamine tetraacetic acid.

Amino acids were extracted from the vesicles three times using 2 ml of 5 % trichloroacetic acid in water each time, the trichloroacetic acid then being removed by shaking with diethyl ether. The solution was evaporated to dryness in evacuated tubes at 50 °C and the residue taken up in 1 ml of 0.1 N HCl. Amino acids were identified by thin-layer chromatography according to Stahl⁸ using 0.25 mm thick Kieselgel G plates. Two-dimensional chromatograms were developed with 70 % ethanol in water and 75 % phenol in water. The dried plates were sprayed with 15 % ninhydrin in 3 M potassium citrate buffer (pH 5.1), and the spots developed for 10 min at 105 °C. The quantitative determination of amino acids was performed using an automatic amino acid analyzer (Hitachi Perkin-Elmer 034 Liquid Chromatograph), employing known standards in ion-exchange chromatography as well as in thin-layer chromatography. Alanine was used as a standard for the unknown compounds. One batch (2 mg) of amino acid extracts was hydrolysed in 6 N HCl for 6 h at 100 °C, and the amino acids in the hydrolysate were determined as above. Quantitative analyses,

Fig. 1. Diagram of a sucrose gradient used for the fractionation of synaptosomal components, and their subcellular particle fractions after the centrifugation.

in addition to being performed in the vesicle fractions, were also carried out in the sample fraction (Fig. 1) and in the synaptoplasm left after the sedimentation of subcellular particles of hypo-osmotically disrupted synaptosomes.

RESULTS

Ten amino acids were identified in the calf brain synaptic vesicles: cysteic acid (or cysteinesulfinic acid), taurine, aspartic acid, glutamic acid, glutamine, serine, alanine, glycine, γ -aminobutyric acid and lysine (for concentrations, see Fig. 4). In addition, the vesicles contained a high concentration of an unknown ninhydrin-positive compound (Figs. 2, 3, 4). Its concentration in the vesicles (in alanine equivalents) was approximately as high as that of all the other amino compounds together (Fig. 4). It was completely hydrolysed in 6 N HCl (Figs. 2, 4) to a variety of amino acids, mainly aspartic acid, glutamic acid and serine (Fig. 4). The vesicles also contained other unknown acid-labile compounds eluting with the cysteic

Fig. 2. Photographs of thin-layer chromatograms of unhydrolysed (A) and hydrolysed (B) amino acid samples from calf brain synaptic vesicles on 0.25 mm thick Kieselgel G run with 70 % ethanol (solvent I) and 75 % phenol (solvent II). The dried plates were sprayed with 15 % ninhydrin, and the spots developed for 10 min at 105 °C. The unknown peptide is indicated by the arrow.

acid peak in ion exchange chromatography (Fig. 4). The concentrations of aspartic acid, glutamic acid and serine, and also the amount of glycine, increased significantly after acid hydrolysis, and minute quantities of some other unknown compounds also appeared (Fig. 4).

The unknown compound was concentrated in the main vesicle fraction (fraction II, Fig. 1, Table 1). Use of the dense sucrose gradient enabled the vesicles to be divided into four fractions, the vesicles of which differed in size, being largest in the lowest fraction, but also more seriously contaminated by unidentified

Fig. 3. Ion exchange chromatograms of a standard amino acid mixture (lower diagram) and an unhydrolysed sample from the synaptic vesicles (upper diagram).

Fig. 4. Quantities ($\mu\text{mol/g}$ vesicle protein) of amino compounds in the vesicles, unhydrolysed and hydrolysed samples. Results (means $\pm$ S.E.M. by vertical bars) are given from 8 determinations.

Table 1. Concentrations of amino compounds ($\mu\text{mol/g}$ protein of the fraction, unknown compounds in alanine equivalents) in the various vesicle fractions, the sample fraction and the synaptoplasm of calf brain cortex.

Amino compound	Concentration of amino compounds in fractions					Synapto- plasm
	I	II	III	IV	Sample fraction	
Cysteic acid	1.0(2)	12.2(24)	0.2(1)	0.3(1)	0.9(1)	2.9(6)
Cysteinesulfinic acid	—	—	—	—	—	0.9(3)
Phosphoethanolamine?	—	—	—	—	—	—
Taurine	3.1(6)	2.4(8)	0.3(1)	0.2(1)	52(4)	148(27)
Aspartic acid	3.4(5)	6.1(11)	2.1(3)	0.8(3)	104(9)	185(9)
Glutamic acid	8.2(19)	14.3(30)	2.5(2)	1.1(4)	156(11)	248(12)
Glutamine	3.8(5)	6.1(8)	1.0(2)	0.9(2)	21(2)	48(4)
Alanine	1.4(3)	2.0(4)	0.2(1)	0.3(0)	9.4(3)	29.5(16)
Serine	3.4(6)	5.8(9)	1.0(1)	0.8(2)	6.1(4)	15.7(12)
Glycine	1.0(2)	1.9(3)	0.3(1)	0.2(1)	16.5(14)	36.5(20)
Lysine	0.2(1)	0.9(1)	0.1(1)	0.1(1)	0.4(1)	1.9(4)
γ -Aminobutyric acid	0.4(1)	3.3(6)	0.2(1)	0.1(0)	77(7)	149(8)
Unknown (peptide) } alanine	5.9(11)	30.5(44)	6.8(10)	4.0(8)	18.1(14)	20.2(15)
Unknown } equivalents	—	—	—	—	2.9(9)	24.0(26)
Cysteine	—	—	—	—	0.6(1)	4.8(3)
Methionine	—	—	—	—	1.2(10)	14.0(10)
Leucine	—	—	—	—	6.3(9)	15.2(11)
Isoleucine	—	—	—	—	0.5(4)	2.8(1)
Tyrosine	—	—	—	—	1.1(2)	6.1(3)
Phenylalanine	—	—	—	—	7.9(6)	12.7(10)
Urea	—	—	—	—	—	1.8(2)
Threonine	—	—	—	—	9.8(10)	10.9(9)
Proline	—	—	—	—	0.2(0.3)	3.1(1)
Valine	—	—	—	—	1.8(2)	5.0(4)
Number of experiments	4	5	3	3	4	5

Means ($\pm$ S.E.M. in parentheses) are given.

arger subcellular particles. Amino acid concentrations were highest in the upper fractions, though their pattern was fairly similar in all fractions (Table 1). These in turn differed considerably from those in the synaptoplasm and sample fractions.

DISCUSSION

The free amino acid composition of the synaptic vesicles presented here agrees fairly well with the figures reported by De Bellerocche and Bradford,¹ Mangan and Whittaker² and Rassin,³ our results being significantly lower only in the case of glutamic acid and taurine. Some loss of intravesicular amino acids certainly occurred during the washing of the vesicles. This stage is of importance, however, for eliminating any extravesicular amino acids which have diffused from the sample fraction into the vesicle fraction.

Several small-molecular peptides have been reported in the central nervous system. "Substance P" contains relatively high amounts of the basic amino acids, arginine and lysine, and of leucine.^{9,10} A large number of glutamate-peptides (glutathione and other γ -glutamyl-peptides), γ -aminobutyrate-peptides (homocarnosine, homoanserine) and lipo- and phospho-peptides have also been found.⁴ An interesting group of central nervous system peptides consists of the *N*-acetylasparyl-peptides,⁴ which have been extensively studied by Reichelt and his associates.^{5,11-13} These peptides are of relatively low molecular weight (4-6 amino acids). They contain *N*-acetylasparyl and several transmitter candidates, some amino acids (including γ -aminobutyric acid and taurine) and also histamine. Two of the most important sequences are *N*-acetylasparyl-seryl-glutamate and *N*-acetylasparyl-glutamyl-seryl-glycine.^{12,13} After hydrolysis, these

peptides thus liberate approximately 30–40 % aspartic acid, 20–30 % serine and glutamic acid and 20 % glycine. The peptide identified in the present paper containing approximately 40 % aspartic acid or *N*-acetylaspatic acid, 30 % serine, 15 % glutamic acid, 10 % glycine and possibly some alanine and lysine after acid hydrolysis, is quite similar to those reported by Reichelt's group. However, there are significant differences regarding the occurrence of histamine, γ -aminobutyric acid and taurine in the peptides demonstrated by Reichelt's group.^{11–13} We were unable to identify these in the hydrolysates of our peptide. The mean amino acid composition also shows certain differences between these peptides. The peptide demonstrated in the present work thus does not seem to be identical with the peptides described earlier, although there are some similarities in their chemical structures. Some of the aspartic acid might also be present in the form of free *N*-acetylaspatic acid.¹⁴

The occurrence of low-molecular peptides in the vesicle fraction is interesting, although their function remains open. Since they contain amino compounds, for which a transmitter role has been proposed, they may function as temporary molecules for storing transmitter substances. The transmitters could then be liberated from them through hydrolysis. This offers a new possibility for terminating the function of transmitter substances in the synaptic region.

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