which confirms the view that these disturbances of metabolism in avitaminosis K or E are secondary in character.

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ISOLATION AND PARTIAL PURIFICATION OF AN ENDOGENOUS INHIBITOR OF ^{3}H-L-GLUTAMATE RECEPTOR BINDING

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KEY WORDS: glutamate receptors; endogenous inhibitors; peptides.

The existence of endogenous substances which inhibit receptor binding of brain neurotransmitters in vitro is very interesting because these substances can claim the role both of true ligands and of modulators of synaptic transmission [7]. Reports on the identification of endogenous inhibitors of GABA [9], benzodiazepine [6], and acetylcholine [10, 12] receptors have recently been published. The hypothesis that there is an endogenous inhibitor of L-glutamate receptor binding was first put forward by Sharif and Roberts [13] in connection with the discovery of increased specific ^{3}H-L-glutamate binding during successive washing of synaptic membranes isolated from brain. The dipeptide N-acetylaspartylglutamate, which inhibits ^{3}H-L-glutamate binding with synaptic membranes, was isolated from a perchlorate extract of rat brain [14]. According to their hypothesis, this isolated dipeptide can perform the function of neurotransmitter in synapses previously classified as glutamergic or aspartatergic. The present writers showed previously [2] that an aqueous extract of synaptosomes contains a thermostable factor of peptide nature, which inhibits specific binding of ^{3}H-L-gluamate.

The aim of the present investigation was to isolate, purify, and determine certain characteristics of this factor.

EXPERIMENTAL METHODS

Specific binding of ^{3}H-L-glutamate (33 Ci/mmole, from Izotop, USSR) with synaptic membranes isolated from the rate cerebral cortex was determined by the method described previously [3].

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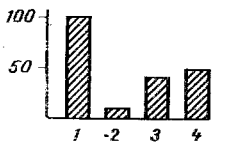

Fig. 1

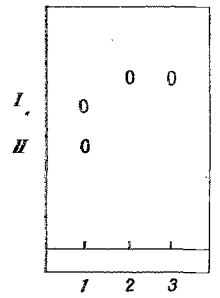

Fig. 2

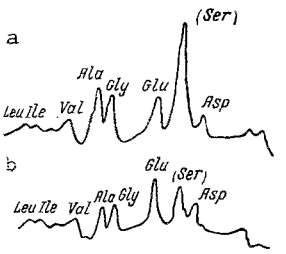

Fig. 3

Fig. 1. Effect of hydrolysis and proteolysis on BIF activity. Ordinate, specific binding (in % of control). Binding of ^{3}H-L-glutamate in control (1), in presence of 5 µl of partially purified fraction of BIF (2), of 5 µl hydrolyzed BIF (3), of 5 µl of BIF subjected to proteolysis (4).

Fig. 2. TLC of partially purified BIF fraction: 1) BIF; 2) BIF after hydrolysis; 3) L-glutamate. I, II) Subfractions.

Fig. 3. Fractionation of subfraction II of BIF on Biotronic LC 6000 amino acid analyzer: a) before, b) after hydrolysis.

The binding inhibiting factor (BIF) was isolated from an aqueous extract of unpurified rat cerebral cortical synaptosomes. The synaptosomes were subjected to osmotic shock by extraction with ice-cold distilled water (1:10 relative to the volume of the fraction) and the membranes were removed by centrifugation (20,000 g, 20 min); the pH of the extract was adjusted to 5.0-5.3 with 85% formic acid and the sample was heated on a boiling water bath for 15 min. Denatured proteins were removed by centrifugation (1500 g, 15 min), the pH of the supernatant was adjusted to 2.0 with formic acid, and the supernatant was applied to a column packed with Dowex AG 50 × 2 resin (the volume of the column for 0.5 l of extract was 25 ml); stepwise elution was carried out with buffer: 0.55 M formic acid, 0.40 M acetic acid, 0.42 M ammonia, pH 3.5. The resulting fraction was evaporated on a rotary evaporator and subjected to gel-filtration on Sephadex G-25 in 0.5 M acetic acid. After testing of the eluate in a radioligand experiment the active fraction was withdrawn and the next stage of purification was carried out by ion-exchange chromatography on Dowex Ag 50 × 2 resin, followed by gradient elution with formate–acetate–ammonia buffer by the method described in [4]. The active fraction of BIF obtained at this stage was desalted on a column with Sephadex G-25 in water.

Thin-layer chromatography (TLC) of the fractions was carried out on plates with Silufol (four-fivefold elution) or with diatomaceous earth (two-threefold elution) in a system of butanol–acetic acid–water (4:1:1). After TLC the plates were developed in ninhydrin or benzidine reagent by the method in [5].

For semipreparative TLC the sample was applied to the plates at the starting line. After TLC the edges of the plate were cut off and devloped. Zones thus identified were scraped off the plates and the test substances were extracted from the carrier with water.

The amino acid composition of the partially purified BIF was analyzed on a "Biotronic LC 6000" amino acid analyzer (the authors are grateful to A. M. Pivovarov and L. F. Arapova, of the Research Institute for General and Special Biochemistry for help with this stage of the experiment).

EXPERIMENTAL RESULTS

As was shown previously, ^{3}H-L-glutamate binds with binding sites of one type on synaptic membranes: K_d (dissociation constant) = 160 nM, B_{max} (the maximal number of binding sites of radioactive label) = 4 pmoles/mg protein [3]. An aqueous extract of synaptosomes actively inhibited specific binding of ^{3}H-L-glutamate: addition of 20 µl of the extract to the standard incubation mixture led to complete inhibition of binding.

The scheme of purification of BIF mentioned above was based on data obtained previously to show that this fact is thermostable, is eluted within the retention volume on gel-filtration on Sephadex G-25, and is retained by the sulfate cation exchange resin Dowex AG 50 × 2 at pH 2.0-3.0. A partially purified fraction of BIF was obtained with the aid of this system. After acid hydrolysis (in 6 N HCl at 110°C for 16 h) or proteolysis with pronase (Sigma, USA)

of this fraction, its ability to inhibit specific binding of ^{3}H-L-glutamate was considerably reduced, but not completely abolished (Fig. 1). It can thus be tentatively suggested that BIF is a peptide containing residues of glutamic (or aspartic) acid, which are set free in the course of hydrolysis and compete with ^{3}H-L-glutamate for binding sites. An alternative hypothesis is that the fraction of partially purified BIF also contains glutamate or aspartate as an impurity. However, the presence of these amino acids in the fraction of partially purified BIF was not confirmed by the results of TLC.

During TLC on plates with silica-gel two subfractions were found in the composition of the partially purified BIF (Fig. 2). Both fractions stained with both benzidine and ninhydrin reagents, evidence that they contained amino acid material. After acid hydrolysis of BIF both subfractions disappeared and a zone with mobility close to that of glutamic acid appeared on the chromatogram.

The results of TLC indirectly confirmed the above hypothesis that bound glutamic acid residues are present in the composition of BIF.

Semipreparative fractionation of BIF by TLC and subsequent analysis of subfractions eluted from the plates on the highly sensitive Biotronic LC 6000 amino acid analyzer showed that the digest of the principal (as regards quantitativbe composition) subfraction II contains the amino acids aspartic and glutamic acids, serine, glycine, and alanine, as well as traces of leucine, isoleucine, and valine. Meanwhile, all the above-mentioned peaks except those of serine and glutamate were found to be identical on the chromatogram of the same fraction before hydrolysis (Fig. 3). The peak corresponding in mobility to serine was greatly reduced, whereas the glutamate peak was increased in amplitude. This may mean that subfraction II included a glutamate-containing peptide, with mobility (in the given system) close to that of serine. There was insufficient material for amino acid analysis of the composition of subfraction I, obtained by semipreparative TLC.

By gel-filtration of the partially purified fraction of BIF on a calibrated column with Sephadex G-25 in 1 M acetic acid, the approximate limits for molecular weight were shown to be 450-600 daltons.

Determination of the properties of BIF by the radioligand method showed that inhibition of specific binding of ^{3}H-L-glutamate is competitive in character.

The experimental results suggest that BIF is a peptide consisting of 2-5 amino acid residues, including residues of glutamic acid. The determination of the precise structure of this peptide will be possible only after it has been purified by more effective methods.

It is doubtful whether BIF is identical with the N-acetylaspartylglutamate mentioned above because several of the effects of the action of this dipeptide described in [14] and, in particular, its effect on receptor binding with glutamate receptors, were not reproduced in subsequent investigations by other workers [8, 11].

Components similar to BIF in mobility during TLC were found in the composition of isolated synaptic membranes and of the glutamate-binding protein solubilized from them and purified by affinity chromatography [1]. Besides indicating that BIF can directly affect the number of active post-synaptic receptors, these data also are evidence of its probable participation in the functioning of glutamatergic brain synapses. It can be tentatively suggested that BIF is an endogenous neuromodulator of glutamate receptors concerned with regulating the efficiency of synaptic transmission.

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ACTION OF ASCORBIC ACID ON BINDING OF ^{3}H-GABA AND ^{3}H-GLUTAMIC ACID TO CEREBRAL CORTICAL SYNAPTOSOMES IN RATS

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KEY WORDS: ascorbic acid, GABA, glutamic acid, cerebral cortex, receptors.

Ascorbic acid (AA) is contained in high and stable concentrations in the mammalian CNS [13]. We know that AA is a coenzyme of dopamine-β-hydroxylase, the enzyme which synthesizes noradrenalin [2] and which also possibly participates in dopamine synthesis [11]. However, concentrations of AA and catecholamines in brain structures do not correlate with each other [10] and AA evidently performs other, as yet unidentified, functions.

Reports of synaptic release of AA [9] and of changes in the firing rate of striatal neurons under the influence of AA [3] suggested that this compound is involved in synaptic transmission. With this suggestion, and also our own observations of the effect of intraventricular injections of AA on Na^+-dependent binding of ^{3}H-GABA [1], in mind it was decided to study the action of AA in vitro on binding of ^{3}H-GABA and ^{3}H-glutamic acid to cerebral cortical synaptosomes. The choice of these mediators was determined by the fact that GABA and glutamic acid are recognized neurotransmitters of cortical neurons [8], and the AA concentration in the neocortex is one of the highest to be found among brain structures [10]. In this investigation Na^+-depending binding, which is effected mainly by presynaptic receptors [15], was studied.

EXPERIMENTAL METHODS

The mitochondrial (synaptosomal) P_2 fraction was obtained from the cerebral cortex of decapitated male Wistar rats. Aliquots of this fraction, each containing 0.4-0.6 mg protein, in 0.1 ml of 0.32 M sucrose solution, were preincubated for 10 min at 0-4°C in 1.9 ml of Krebs–Ringer solution with or without L-AA (10^{-6}-10^{-3} M), after which ^{3}H-GABA (specific radioactivity 1.3 TBq/mmole, from Izotop, Leningrad) or ^{3}H-DL-glutamic acid (specific radioactivity 1.2 TBq/mmole, from Izotop) in a concentration of 10^{-7} M was added. Incubation was stopped after 10 min by centrifugation at 16,000 g and 4°C for 10 min. After washing twice with 50 mM Tris-HCl buffer (pH 7.4) the residue was dissolved in 0.1% Triton X-100 solution and the level of radioactivity was measured in Tritosol [4] on an Isocap-300 scintillation counter (Beckman, USA). Nonspecific binding was determined in the presence of 10^{-3} M unlabeled GABA or DL-glutamic acid; specific binding was calculated as the difference between total and nonspecific. The protein concentration was measured by Lowry's method [7].

EXPERIMENTAL RESULTS

The AA concentration in the incubation medium had a significant effect on Na^+-dependent binding of ^{3}H-GABA (Table 1). With low AA concentrations (from 10^{-6} to 10^{-5} M) specific binding of ^{3}H-GABA was doubled. A further increase in the AA concentration to 10^{-3} M steadily

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