PARTIAL PURIFICATION OF ENDOGENOUS HEPATIC (+)-[^{3}H] SKF 10047 BINDING TO SIGMA OPIOID RECEPTORS

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Opioid receptors are subdivided into four types: μ, δ, κ, and σ. In the CNS of several mammals the σ-opioid receptors have been characterized biochemically [4, 8, 10]. It has recently been shown that these receptors exist in rat liver [1]. σ-Opioid receptors are the only type of opioid receptors for which an endogenous ligand has not been found, although attempts have been made to isolate it from brain extract [9, 11].

The aim of this investigation was to discover endogenous substances in the liver, by a radioreceptor method, capable of interacting with σ-opioid receptors.

EXPERIMENTAL METHOD

Endogenous substances were isolated from hog liver by extraction with acidified acetone followed by precipitation of the protein-peptide fraction by increasing the acetone concentration to 96% [5]. Freshly frozen hog liver (30 g) was homogenized and extracted with 87% acetone solution (138 ml), containing HCl (3 ml), for 1 h at 4°C. The residue was removed by centrifugation at 2000g. The extract (E_1, 103 ml) was added with mixing to acetone (515 ml), cooled to -5°C, and allowed to stand overnight at 4°C. The precipitate thrown down (acid acetone powder — AAP) was separated by centrifugation at 2000g, washed with acetone, and dried in a current of nitrogen. The acetone was removed from the supernatant in vacuo and an equal volume of an ether–hexane mixture (2:1) was added to the remaining aqueous solution and the lipids were extracted by shaking in a separating funnel. The upper layer and the darkly colored interphase were discarded and the bottom layer (E_2) was used in the subsequent work. A 10% solution of trifluoroacetic acid (TFA) was added to E_2 up to a concentration of 0.1%, and activity was concentrated on "Sep-Pak C_{18}" minicolumns (Waters, USA) by the method in [3]. Solution with low activity passing through was discarded, whereas the active eluates (E_3) were evaporated in vacuo. A 1/20 part of E_3 was then fractionated by high-efficiency liquid chromatography (HELC) on 4.6 × 250 mm "Si-100 Polyol" (Serva, West Germany) and 4.0 × 300 mm "μ Bondapak" C_{18} (Waters) columns, fitted with preliminary columns measuring 4.6 × 40 mm. An "Altex" 322 MP chromatograph (USA) and a "Uvicord SD" spectrophotometric detector (LKB, Sweden) were used. The optical density of the eluate was measured at a wavelength of 226 nm. Different fractions of AAP were obtained by passing it through

TABLE 1. Yield and Radioreceptor Activity of AAP and E_2 Fractions (per gram liver tissue)

Fraction	Weight, mg	Protein, mg	Radioreceptor activity	
			specific, nU/mg	total, nU
AAP	7,7	3,7	0,008	0,06
E_2	25	0,3	0,8	20

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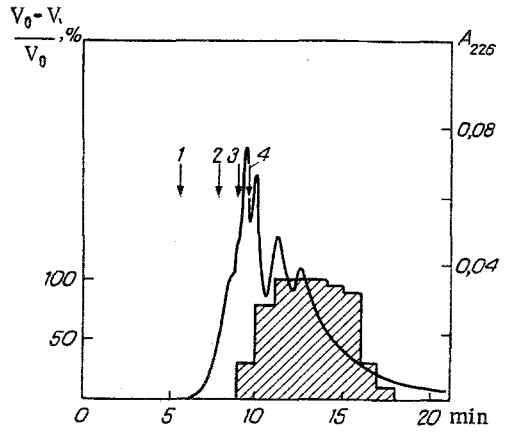

Fig. 1. Fractionation of E_3 on column (4.6 × 250 mm) with "Si-100 Polyol" sorbent. Eluent: 50 mM Tris-HCl (pH 7.2), containing 100 mM KCl and 40% acetonitrile. Rate of flow 0.5 ml/min. E_3 added in a volume of 50 µl in initial buffer. Columns indicate radioreceptor activity. Arrows indicate retention time of marker substances: 1) bovine serum albumin, 2) insulin β-chain, 3) alanine.

a "Sep-Pak C_{18}" minicolumn, followed by elution with 0.1% TFA (AAP-0.1) and 10% (AAP-10), 20% (AAP-20), 50% (AAP-50), and 80% (AAP-80) solutions of acetonitrile in 0.1% TFA. Protein in the fractions was determined by the reaction with the dye Coomassie G-250 [6]. Aliquots of the fraction were tested for their ability to inhibit binding of (+)-[^{3}H] SKF 10047 (41.8 Ci/mole; "New England Nuclear," USA), a ligand of σ-opioid receptors, with rat liver membranes. The liver membranes were obtained by the method in [7]. 200 µl of the resulting membrane suspension, containing 400 µg protein, and 100 µl of liver extract fraction were incubated for 45 min at 25°C with 1 nM (+)-[^{3}H] SKF 10047 in the presence of 10 µg/ml of bestatin. At the end of incubation the samples were quickly filtered through GF/C glass filters ("Whatman," England) and the tubes were sprinkled with incubation buffer (1 ml twice), and the filters washed with the same buffer (4 ml 3 times). The quantity of bound ligand was estimated by measuring radioactivity by means of an LS 6800 liquid scintillation counter ("Beckman," USA). Nonspecific binding was determined in the presence of 10 µM SKF 10047. Activity of the liver extract fractions was pressed in terms of IC_{50} — the quantity of extract inhibiting binding of (+)-[^{3}H] SKF 10047 by 50%, and also in nanounits (nU). Activity equivalent to 1M SKF 10047 was taken as the unit of activity. The E_4 fractions after chromatography on a "µ Bondapak C_{18}" column also were tested for their ability to interact with rat brain σ-opioid receptors and with rat vas deferens opioid receptors. Interaction with σ-opioid receptors was estimated as inhibition of binding of 3 nM [^{3}H]D-Ala^2-D-Leu^5-enkephalin ([^{3}H] DADLE; 45 Ci/mmole) with brain membranes. The [^{3}H] DADLE was obtained from D. Zaitsev (Institute of Molecular Genetics, Academy of Sciences of the USSR). Nonspecific binding of [^{3}H] DADLE was determined in the presence of 500 nM of unlabeled DADLE. The effect on contraction of the isolated vas deferens was studied in thermostatically controlled cells (36°C) during stimulation by an electric field with pulses 1 msec in duration and with a frequency of 0.1 Hz, at supramaximal intensity. The test fractions were introduced into the cells in a volume of 5 and 25 µl 3 min before addition of β-endorphin (100 nm). To establish the nature of the active material, E_3 was treated with pronase (Calbiochem-Behring Corp., West Germany). 400 µl of E_2 in 50 mM Tris-HCl buffer was incubated with 10 µg of pronase for 30 min at 40°C.

The reaction was stopped by boiling for 5 min and radioreceptor activity of the pronase-treated extract was determined. A sample of E_3 without pronase and a solution of pronase itself (10 µg to 400 µl of buffer), treated by the same method, were used as the control.

EXPERIMENTAL RESULTS

Data showing the yield and radioreceptor activity of two fractions obtained from hog liver — AAP and acetone supernatant E_2 — are given in Table 1. It can be seen from the data that the greater part of the activity is concentrated in E_2. The AAP, containing the

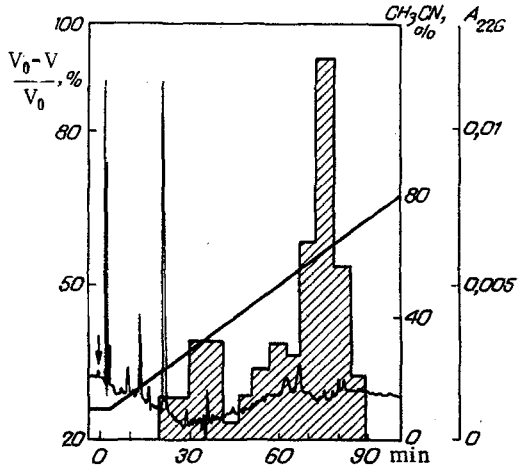

Fig. 2. Reversed-phase chromatography of active fraction E_4 on "μ Bondapak C_{18}" column. Eluent A: 0.1% TFA in 10% acetonitrile; eluent B: 0.1% TFA in 80% acetonitrile, linear gradient of B and A for 90 min. Rate of flow 1 ml/min. Altogether 50 μl of E_4 in eluent A was added.

main part of the protein-peptide substances of the extract, exhibited activity in doses more than 250 times greater than IC_{50} for E_2 (500 and 2 mg respectively of the original tissue). When the AAP was passed through the "Sep-Pak" minicolumn, its activity was determined, starting with the AAP-20 fraction (42% of inhibition), and maximal values were reached in fractions AAP-50 and AAP-80 (94 and 90% inhibition respectively).

Since the E_2 fraction possessed greater specific activity than AAP, it was subjected to further purification. The results of high-efficiency gel chromatography of the E_3 fraction on a "Si-100 Polyol" column are shown in Fig. 1. The active material was eluted from the column after alanine (mol. wt. 89 daltons), indicating interaction of the active substances with the material used to pack the column, and it was thus impossible to estimate their true molecular weight. A similar picture was observed after gel filtration of E_3 on Sephadex G-25.

The results of further purification of the active fraction E_4 by reversed-phase chromatography are shown in Fig. 2. Activity was found in two fractions with retention times of 30-40 min and 70-75 min. The two fractions were eluted in the presence of high concentrations of acetonitrile (about 36 and 66% respectively), evidence of the high lipophilicity of the active substances. It will be noted that none of the fractions obtained by reversed phase chromatography or the initial E_4 inhibited binding of [^{3}H] DADLE with rat brain membranes. Meanwhile the fractions in these doses effectively inhibited binding of (+)-[^{3}H] SKF 10047 with liver membranes. These fractions also did not change the amplitude of evoked contractions of the vas deferens and did not affect the magnitude of the inhibitory effect of β-endorphin. Incidentally, the specific ligand of σ-opioid receptors (+)-bremazocine, under these same conditions, likewise was inactive. The results indicate that compounds interacting selectivity with σ-opioid receptors are present in the liver. So far as the chemical nature of the active substances of the E_2 fraction is concerned, it can be tentatively suggested that it is a compound of nonpeptide nature, for incubation with pronase did not lead to any loss of activity. The high thermostability of the active material of the extracts must be noted. Activity was completely preserved after boiling of a solution of E_3 for 5 min.

We thus showed for the first time that liver tissue contains substances interacting selectively with σ-opioid receptors. These substances are contained in small quantities in the protein-peptide fraction of liver extract and are concentrated in its acetone supernatant. Substances of the acetone supernatant posses high lipophilicity and thermostability, they are not destroyed by treatment with pronase and, evidently, they are not peptides. It can be tentatively suggested that endogenous ligands of σ-opioid receptors, whose possible role in the CNS and visceral organs was examined by the writers previously [2], are contained in the liver.

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EFFECT OF INTRAUTERINE EXPOSURE TO ETHANOL ON POSTNATAL SYNTHESIS OF SOME RAT BRAIN PHOSPHOLIPIDS

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According to clinical observations, a fetal alcoholic syndrome, a disease accompanied by disturbances of physical and mental development and by impairment of the mental faculties, is observed frequently in children whose mothers abuse alcohol during pregnancy [5]. It has been shown that animals exposed to alcohol in the intrauterine period are retarded in height and weight [14], their ability to form and maintain conditioned reflexes is reduced [6], and energy and protein metabolism in the brain is disturbed [3, 11]. Meanwhile brain lipid metabolism in inborn cerebral pathology of alcoholic genesis has so far received little study. We know that ethanol, on penetrating from the blood stream into the brain, acts on the lipid components of the cell membranes and modifies their permeability [12]. It is very probable that chronic exposure of the undeveloped brain to ethanol may prevent the normal formation of the lipid composition of the membrane. It was accordingly decided to study parameters of phospholipid synthesis in the brain of rats exposed antenatally to ethanol.

We know that when the aftereffects of intrauterine damage to the CNS are studied, the use of additional provocative factors can lead to the detection of latent metabolic shifts [1]. One aspect of the present investigation was therefore a study of the features of phospholipid synthesis in response to functional loading.

EXPERIMENTAL METHOD

Female albino rats were given ethanol perorally in a daily dose of 5-7 g/kg from the 1st day of pregnancy and until giving birth, whereas control animals received water. The male progeny of these mother rats, at the age of 2 months, were lightly anesthetized with ether and given an injection of sodium ^{32}P-orthophosphate (0.4 MBq in a volume of 50 µl) into the left lateral cerebral ventrical. The action of the anesthetic continued for 10-12 min. The rats were decapitated 90 min after injection of labeled phosphate. In some experiments, for 1 h before sacrifice the animals were subjected to functional loading in the form of vibration and noise in a metal box.

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