ACTION OF ANTITHEINES WITH DIFFERENT MNEMIC EFFECTS ON cAMP-INDEPENDENT PROTEIN KINASES OF BRAIN CHROMATIN

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Among the structural analogs of the memory stimulator ethylnorantitheine (ethimizole) compounds not only with a positive (its demethylated derivatives), but also with a negative (allyl- and propylnorantitheines) effect on preservation of an elaborated skill have been isolated [1, 2]. The study of the molecular mechanisms of action of these antitheines has revealed definite correlation between their mnemic effects and their direct action on the RNA-synthesizing activity of rat brain neurons [6]. It has been suggested that their regulatory influence on the genetic apparatus is mediated through cAMP-independent protein kinases of chromatin [5]. In that case, changes in structure of the ethimizole molecule, of significance for its effects on memory, ought also to be manifested in the action of these enzymes. The present communication is devoted to an examination of this problem.

EXPERIMENTAL METHOD

Male albino rats weighing 200-220 g were used. Nuclei and chromatin were isolated from rat cerebral cortical neurons as described previously [3, 5].

cAMP-independent protein kinases I and II were extracted from chromatin (10 mg protein) with 0.3 M $(NH_4)_2SO_4$ in buffer A, in conjunction with ultrasonic disintegration. Buffer A consisted of: 50 mM Tris-HCl, pH 7.9, 25% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 5 mM β-mercaptoethanol, and 5 mM benzamidine. The extract was centrifuged for 1 h at 100,000*g*. The supernatant was dialyzed overnight against 0.1 M NaCl in buffer A, and then centrifuged for 20 min at 20,000*g*. Protein kinases I and II were separated from the supernatant by ion-exchange chromatography on a column (10 × 0.9 cm) with DEAE-Sephadex A-25 in a linear concentration gradient from 0.1 to 0.35 M NaCl in buffer A. The enzymes thus obtained were purified by gel-filtration on a column (50 × 1 cm) with Sephadex G-150, with 0.35 M NaCl in buffer A. Eluates with protein kinase activity were pooled and used for further investigation. All procedures were carried out at 4°C.

The incubation mixture (15 min, 30 sec) for determination of protein kinase activity contained (in a volume of 250 μl): 50 mM Tris-HCl, pH 7.8; 10 mM $MgCl_2$; 10 mM β-mercaptoethanol; 100 μg of protein substrate; 100 μM ATP; 50 μl of enzyme; 0.37 MBq ^{33}P-ATP (60-90 PB/mole) and the necessary quantity of test preparations. The reaction was stopped on ice by the addition of an equal volume of cold 30% TCA containing 10% Na pyrophosphate and 0.5 mM ATP, transferred to "nuclear" filters (d = 0.2 μ), and washed with 25% TCA. Radioactivity of the filters was measured in toluene scintillator on a RackBeta counter (LKB, Sweden).

Total HMG-protein was obtained by extraction with 5% perchloric acid from rat liver nuclei [7]. Protein was determined by the method in [8]. The results were subjected to statistical analysis by a special computer program for calculating Student's "t."

EXPERIMENTAL RESULTS

The level of phosphorylation of proteins of rat brain neuron chromatin in the presence of ^{33}P-GTP (conditions specific for determination of cAMP independent protein kinase II) exceeded the level of this process in glial cells by 1.5 times (0.443 ±

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TABLE 1. Effect of Antitheine on Activity of cAMP-Independent Protein Kinase II (nmoles ^{33}P/ml/h) from Rat Brain Neuron Chromatin (M ± m, n = 5)

Substance	0	Concentration of substances, M			
		10^{-8}	10^{-7}	10^{-6}	10^{-5}
Norantitheine	257±1,1	261,5±1,8	222,8±5,8	223,2±6,4	—
M_1		344,0±2,7*	477,0±7,4*	378,0±5,9*	245,0±4,2
M_2		383,0±1,6*	439,6±3,2*	365,0±2,1*	268,0±5,8
Ethylnorantitheine		398,0±8,0*	415,0±6,8*	350,0±5,5*	260,0±2,2
Allylnorantitheine		174,0±2,3*	134,0±2,5*	243,0±3,8	249,0±4,9
Propylnorantitheine		257,0±1,5	243,0±2,1	237,0±0,9	—

Legend. *$p < 0.05$ compared with control.

0.0009 and 0.304 ± 0.0009 nmoles/mg protein/h respectively). ethimizole, in a concentration of 10^{-5} M significantly increased (by 15%), whereas allylnorantitheine reduced (by 12%) the protein kinase activity of the neurons. Under these circumstances the preparations did not affect phosphorylation of glial cells in the chromatin. Incidentally, ethimizole had no action on RNA synthesis in glia [3].

To study the character of action of ethimizole and its analogs, two cAMP independent protein kinases were isolated from chromatin of rat brain neurons, and on the basis of a series of properties they were identified as types I and II casein kinase (PKI and PKII). It was shown previously that ethimizole increases the phosphorylation of chromatin proteins on account of the fraction of nonhistone HMG-like proteins, which play an important role in the regulation of transcription [5]. We therefore isolated from the nuclei the fraction of total HMG-proteins and used it as phosphorylation substrate for PKI and PKII.

The experiments demonstrated the selectivity of action of preparations of the antitheine series relative to PKII, for they were found to have no significant effect on activity of cAMP-independent PKI (on average it was 11.5 ± 0.1 nmole ^{33}P/ml/h). Meanwhile activity of PKII was significantly increased by the action of ethimizole in various concentrations (from 10^{-8} to 10^{-6} M) (Table 1). As was mentioned above, its demethylation with respect to one (in M_1) or two (M_2) methylcarbamoile groups does not lead to loss of ability to stimulate activity of the genetic apparatus of the nerve cells or long-term memory [2, 4]. In the present study the demethylated analogs (M_1 and M_2), like the original substance, also potentiated activity of the enzyme in a wide range of concentrations. The maximal effect was observed in M_1: in a concentration of 10^{-7} M it increased phosphorylation of proteins by 83% (M_2 by 71%, ethimizole by 62%). Norantitheine, which has low pharmacological activity, had no effect on PKII. By contrast with demethylation, increasing the weight of the radical in position 1 of the imidazole ring of allylnorantitheine led to reduction of the RNA-synthesizing activity and to an increase in the skill deficit in various models of learning [1]. The preparations showed a similar action also on PKII: in a concentration of 10^{-7} M activity of the enzyme was reduced by 48% compared with the control. Replacement of the unsaturated (allyl) radical by a saturated in propylnorantitheine, moreover, abolished the effect.

It was thus shown that ethimizole and its structural analogs may have a direct action on cAMP-independent PKII. Changes in the level of phosphorylation of regulatory nonhistone proteins in this case correlate to a definite degree with the character of changes in the template activity of chromatin. This very convincingly confirms our suggestion that cAMP-independent protein kinase II of rat brain neuron chromatin is the target for the action of these compounds in the realization of their mnemic effects.

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PHOSPHATASE ACTIVITY IN THE LYMPH DURING THE FEBRILE REACTION

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In previous investigations we showed that the enzyme composition of the lymph in various pathologies (shock, allergy, terminal states, postresuscitation period, inflammation) reflects changes in cell membrane permeability and the degree and depth of cellular injuries more accurately than blood enzyme levels. On the other hand, we know that the febrile reaction (FR) is accompanied by considerable changes in enzyme activity in various organs and tissues and in the blood serum. Meanwhile the character of changes in enzyme activity in lymph flowing from different organs and regions of the body and their comparison with changes in blood enzyme activity during FR have not been studied.

We accordingly undertook a comparative investigation of activity of acid (AcP) and alkaline (AlP) phosphatases and of AlP isozymes in lymph and blood in the course of FR of varied duration.

EXPERIMENTAL METHOD

Experiments were carried out on 64 chinchilla rabbits weighing 2.5-4.2 kg. FR was produced by injecting pyrogenal by the method described previously [8]. Animals receiving an injection of pyrogen-free physiological saline, made up in bidistilled water, served as the control. Lymph for determination of activity of AcP [11], AlP [10], and its isozymes — isolated from liver (AlP_{liv}), intestine (AlP_{int}), and bone (AlP_{bone}) was obtained from the thoracic lymph duct (TLD) and the hepatic lymphatic trunk, while blood for investigation was taken from the femoral vein. The experimental results were subjected to statistical analysis. The animals were killed humanely by injection of a lethal dose of general anesthetic.

EXPERIMENTAL RESULTS

The investigations showed (Tables 1-3) increased activity of total AlP in both types of lymph in the course of FR. With an increase in the duration of fever, the degree of activation of the enzymes increased, although the degree of increase of AlP activity in lymph flowing from the liver was lower than in lymph from TLD. The degree of the increase in the total AlP level in the blood was less, and its normal level was regained sooner, than in lymph (5-5.5 and 10 days after 1 and 3 injections respectively of pyrogenal).

The AlP isozyme spectrum in the biological fluids changed on the whole similarly to total activity of the enzyme. However, the increase in activity of the intestinal isozyme in the lymph was greater than that of the liver and bone isozymes. Meanwhile, whereas an increase in the content of AlP_{liv} and AlP_{int} in lymph from TLD was observed at all times of investigation, the AlP_{bone} level in the late stages after 3 and 5 injections of pyrogenal returned close to its original values. The increase in the content of individual isozymes in the blood was inconstant and was less than in the lymph.

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