eral influences to the efferent component of the respiratory system [1-3]. Comparison of the intensity of the changes in EMG of RN to ipsilateral and contralateral cortical stimulation showed that the character of and relationship between asymmetry of RM activity before and after callosotomy were the same as for RN (reflecting to some degree the processes taking place in RC).

The corpus callosum, which maintains functional relations between each hemisphere and the ipsilateral and, more especially, the contralateral halves of RC, thus participates in the mechanisms of paired activity of RC. This participation is manifested by the fact that the corpus callosum determines the number of responding RN of the right and left halves of RC, and also the character of their responses to cortical stimulation.

LITERATURE CITED

1. T. I. Goryunova, Byull. Éksp. Biol. Med., No. 11, 30 (1961).
2. T. L. Zhigailo, Fiziol. Zh. (Ukr.), No. 2, 167 (1980).
3. A. D. Kocherga, in: Proceedings of the 10th Congress of the I. P. Pavlov All-Union Physiological Society [in Russian], Vol. 2, Moscow–Leningrad (1964), p. 423.
4. N. A. Merkulova, "Character and causes of development of asymmetry and other disturbances of respiration," Author's Abstract of Doctoral Dissertation, Kuibyshev (1965).
5. N. A. Merkulova, and N. L. Mikhailova, Byull. Éksp. Biol. Med., No. 11, 528 (1981).
6. N. A. Merkulova and B. Ya. Peskov, Fiziol. Zh. SSSR, 47, 178 (1961).
7. E. V. Montsevichyute-Éringene, Patol. Fiziol., No. 4, 71 (1964).
8. V. A. Safonov, E. N. Efimov, and A. A. Chumachenko, Nauch. Dokl. Vyssh. Shkoly, Biol. Nauki, No. 8, 118 (1968).
9. M. V. Sergievskaya and N. Ya. Kireeva, Byull. Éksp. Biol. Med., No. 12, 632 (1980).
10. M. V. Sergievskii, N. A. Merkulova, R. Sh. Gabdrakhmanov, et al., The Respiratory Center [in Russian], Moscow (1975).
11. M. Bassal, A. L. Bianchi, and M. Dussardier, J. Physiol. (Paris), 77, 779 (1981).
12. E. K. Bystrzycka, Brain Res., 185, 59 (1980).
13. M. Kalya, J. Feldman, and M. I. Cohen, Brain Res., 171, 135 (1979).
14. J. Lipski and E. G. Merril, Brain Res., 197, 521 (1980).
15. E. G. Merril, Fed. Proc., 40, 2389 (1981).

PROTHROMBIN — THE SUBSTRATE FOR BLOOD PLASMA KALLIKREIN AND FACTOR XIIa

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KEY WORDS: prothrombin; kallikrein; factor XIIa; blood plasma.

The blood clotting system is a cascade of successive reactions of activation of proenzymes (inactive clotting factors) into the corresponding enzymes (active clotting factors) and it is postulated that each enzyme can activate only one, strictly definite, specific precursor protein [9]. However, many recent investigations have shown that this principle is infringed. Limited proteolysis of the same proenzymes can take place by various enzymes, not only of the blood clotting system, but also of the fibrinolytic, kallikrein-kinin, and complement systems which are functionally closely connected with it [15].

It is accordingly important to study the action of the various blood plasma proteolytic enzymes on prothrombin, the precursor of the enzymes thrombin, and to assess the possibility of generation of thrombin activity under these circumstances. We know that factor Xa is a

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TABLE 1. Clotting and Esterase Activity of Prothrombin and Products of Its Proteolysis by Plasma Kallikrein Preparations and Factors α-XIIa and β-XIIa Immobilized on Enzacryl

Test	Prothrombin +													
	physiological saline						kallikrein						α-XIIa	β-XIIa
	experiment I					expt. II	experiment I					expt. II		
	incubation time, h													
	0	24	48	72	96	75	0	24	48	72	96	75	72	72
Prothrombin activity, percent of initial level	100	73	63	56	51	40	100	25	15	12	10	9	16	200
Thrombin activity, min	∞	∞	∞	∞	∞	∞	∞	80	52	25	27	25	25	25
Clotting time in presence of thrombin, sec	49	49	49	49	49	49	49	30	24	16	14	13	21	20
BAME-esterase activity, μmoles/BAME/mg protein/min	0	0	0	0	0	0	0	1,5	2,8	5,6	—	3,0	2,25	19,5

specific activator of prothrombin. Plasmin does not stimulate generation of thrombin activity; on the contrary, it hydrolyzes prothrombin with the formation of products possessing antithrombin activity and capable of inhibiting activity of factor Xa [5]. As a result of the proteolytic degradation of prothrombin by blood plasma kallikrein, thrombin activity was observed to appear [2].

The investigation described below was devoted to a further study of proteolysis of prothrombin by human blood plasma kallikrein and active factor XII, also obtained from human blood plasma. These enzymes were first immobilized on Enzacryl-AH to enable their complete separation from the reaction products at the end of proteolysis.

EXPERIMENTAL METHOD

Prothrombin was isolated from bovine plasma and purified by chromatography on DEAE-cellulose [6]; its activity was determined by a two-stage method in the absence and in the presence of added extrinsic factor X [10]. Factor X was obtained as a by-product during prothrombin purification. Kallikrein was obtained from human blood plasma by the method in [3] and was immobilized on Enzacryl-AH [8]. Factor XII was isolated from human blood plasma and activated with glass for 24 h at room temperature [4], and surface-activated factor α-XIIa was obtained as a result. To obtain the fragment of active factor XII, surface activation with glass was continued for 5-6 days and factor β-XIIa was isolated from the activation products by preparative polyacrylamide gel electrophoresis [4]. Both preparations were immobilized on Enzacryl-AH [8]. Activity of factors α-XIIa and β-XIIa was determined indirectly by their ability to activate human blood plasma prekallikrein into kallikrein, by the method in [4]. Activity of the XIIa factors was estimated according to the BAEE*-esterase activity [14] of the kallikrein thus formed. This activity was 0.121 μmole BAEE/mg dry weight of the immobilized preparation of factor α-XIIa/min and 0.060 μmole BAEE/mg immobilized factor β-XIIa/min. The BAME†-esterase activity [14] of the immobilized kallikrein was 0.036 μmole BAME/mg dry weight of the preparation/min. The thrombin amidase activity of products of proteolysis of prothrombin by kallikrein was determined on chromogenic substrate S-2238,‡ generously provided by Dr. A. Söderberg (Kabi Diagnostica, Sweden) at 405 nm on a Hitachi-124 spectrophotometer according to the method in [13]. Clotting activity was determined from the time of formation of a fibrin clot after mixing equal volumes of 0.3% fibrinogen solution and the test samples (37°C). Antithrombin activity was determined in a system consisting of 0.1 ml of 0.3% fibrinogen solution, 0.1 ml of the test preparation, and 0.1 ml thrombin (2 mg in 1 ml physiological saline). The test samples were replaced by physiological saline in the control tests. Fibrinogen and thrombin were from the Kaunas Bacterial Preparations Factory, and the BAEE and BAME were from Reanal, Hungary.

*BAEE — N-benzoyl-arginine ethyl ester.
†BAME — N-benzoyl-arginine methyl ester.
‡S-2238 — H-D-Phe-Pip-Arg-pNa.

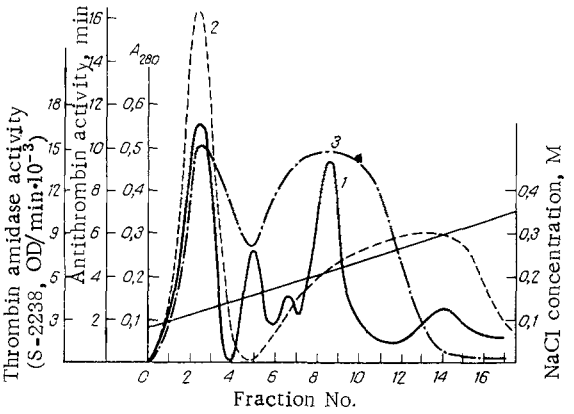

Fig. 1. Fractionation of prothrombin preparation on column with DEAE-Sephadex A-50 after incubation for 75 h with plasma kallikrein immobilized on Enzacryl-AH. Curves: 1) A 280, 2) antithrombin activity, 3) thrombin amidase activity.

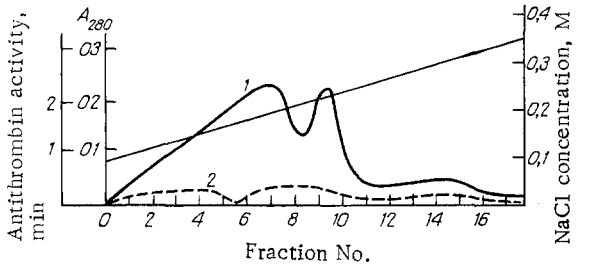

Fig. 2. Fractionation of prothrombin preparation on column with DEAE-Sephadex A-50 after incubation for 75 h with physiological saline. Legend as to Fig. 1.

EXPERIMENTAL RESULTS

Prothrombin preparations uncontaminated by factor X, in concentrations of 0.014 and 0.024 mg/ml with activity of 2500 and 3000 NIH units/mg protein in 0.02 M citrate buffer, pH 7.0, containing 0.1 M NaCl, were incubated with an equal volume of immbolized kallikrein with constant shaking at room temperature. Proteolysis was stopped by removing the immobilized kallikrein by centrifugation.

In one of the two experiments proteolysis of prothrombin continued for 75 h, and in the other the samples were analyzed after incubation for 0, 24, 48, 72, and 96 h. In control experiments prothrombin was incubated with physiological saline under the same conditions. In the control and experimental samples prothrombin, clotting, and BAME-esterase activities were determined. The results given in Table 1 show that proteolysis of prothrombin by immobilized kallikrein was accompanied by a fall in the prothrombin level and by the appearance of considerable esterase and weak clotting activity. Shortening of the fibrin clot formation time by the addition of thrombin from an external source also was noted.

In the next experiment a prothrombin preparation with lower activity (700 NIH units/mg protein) was used. After incubation with immobilized kallikrein of the same activity as in the previous experiments for 75 h, no intrinsic clotting activity could be detected, evidently because of the lower activity of the original prothrombin. Meanwhile, just as in the previous experiments, the fibrinogen clotting time was shortened by the addition of external thrombin from 49 to 20 sec. The proteolysis products were then fractionated on a column with

DEAE-cellulose. The proteolysis products were eluted with 0.025 M citrate buffer, pH 7.8, in an ionic strength gradient from 0.08 to 0.35 M NaCl (Fig. 1). Several fractions possessed high antithrombin activity, and for some of them it was 16 min compared with 50 sec in the control. The antithrombin activity of the fractions was directed toward inhibition of aggregation of fibrin monomers and not proteolysis of fibrinogen by thrombin, for inhibition of thrombin amidase activity was not observed relative to the chromogenic substrate S-2238 in the presence of these fractions. Against the high background of this antithrombin activity no appreciable clotting activity could be detected. The distribution of intrinsic thrombin amidase activity relative to thrombin substrate S-2238 among the fractions is shown in Fig. 1. The value of the apparent K_m for fraction I was determined on the same substrate S-2238. It was found to be $38 \cdot 10^{-5}$ M, considerably more than K_m for α-thrombin ($0.9 \cdot 10^{-5}$ M) [7]. This fact suggested that in this case amidase activity was due, not to thrombin, but to its active precursor meizothrombin, which is characterized by weak clotting activity but by sufficiently well-marked amidase activity and which differs from thrombin in its longer A-chain [11]. The results of fractionation of the prothrombin preparation, preincubated beforehand with physiological saline at room temperature for 75 h, under the same conditions are illustrated in Fig. 2. After fractionation neither amidase nor antithrombin activity was observed in the products.

Next, 8 ml of a solution of factor X (concentration 0.3-0.4 mg/ml) in 0.025 M citrate buffer, pH 7.55, containing 0.5 M NaCl, was incubated with 1.5 ml of factor α-XIIa (surface-activated) or factor β-XIIa (fragment), immobilized on Enzacryl-AH, at room temperature with constant shaking. Kallikrein immobilized on Enzacryl-AH in one experiment was added in a volume of 7 ml to factor X, in the other in a volume of 5 ml. In the first experiment the pH of the medium was 7.75, in the second experiment 8.0. The reaction products were analyzed after removal of the immobilized enzymes by centrifugation. In the control experiment factor X was incubated with physiological saline. Neither in the control nor in either experiment was the appearance of BAME-esterase activity observed, evidence of the absence of conversion of factor X into factor Xa under these experimental conditions. Meanwhile this preparation of factor X was converted into factor Xa during two-stage determination of prothrombin activity. Factor X stimulated activation of highly purified prothrombin preparations in the presence of thromboplastin, Ca^{++}, and factor V, so that activity of the thrombin preparations completely free from contamination with factor X could be determined. These findings are evidence that the preparation of factor X which we used was in the native state.

The prothrombin preparation was used in a concentration of 0.015 mg/ml, containing factor X as an impurity, with activity of 1250 NIH units/mg protein. A solution of prothrombin in 0.02 M citrate buffer, pH 7.75, containing 0.1 M NaCl, was incubated with shaking for 72 h at room temperature with immobilized factor α-XIIa and β-XIIa. The same preparation of prothrombin, incubated under the same conditions with physiological saline, was used as the control. The proteolysis products were analyzed after removal of the immobilized enzymes by centrifugation. Table 1 shows that compared with the control there was a sharp fall in the prothrombin level after incubation with surface-activated factor α-XIIa. Under these circumstances considerable BAME-esterase activity and weak clotting activity appeared. Further evidence of the increase in the clotting potential also was given by shortening of the clotting time of fibrinogen by thrombin in the presence of the proteolysis products. A similar effect also was observed as a result of incubation of prothrombin with immobilized factor β-XIIa. However, the increase in BAME-esterase activity under these circumstances was almost ten times greater than the increase caused by factor α-XIIa. Prothrombin activity was doubled in the presence of added extrinsic factor X. To explain this phenomenon it can be postulated that products arising during proteolysis sharply intensify activation of factor X into factor Xa which, in turn, is a direct activator of prothrombin [15]. Differences in the mechanisms of proteolysis of prothrombin by factor α-XIIa and factor β-XIIa are further evidence in support of the view that these enzymes differ as regards hydrolysis both of certain low-molecular-weight substrates and of the original factor XII molecule during autolysis [12].

The results of the present investigation and also those published by the writers previously [1, 5] lead to the conclusion that prothrombin is a substrate not only for factor Xa, but also for plasmin, plasma kallikrein, and factor XIIa. It is also probable that one cause of thrombotic complications which accompany some pathological states characterized by elevation of the plasma kallikrein level may be the thrombin activity of products of proteolysis of prothrombin by kallikrein. In this connection it is very important that neither kalli-

krein nor factor XIIa nor plasmin [1] can activate factor X and, consequently, in such situations the possibility that activation of prothrombin into thrombin by these enzymes takes place indirectly, through its physiological activator factor Xa, must be ruled out.

LITERATURE CITED

1. I. P. Baskova and E. G. Mirkamalova, Biokhimiya, 44, 1286 (1979).
2. I. P. Baskova, E. G. Mirkamalova, G. A. Yarovaya, et al., Biokhimiya, 44, 2253 (1979).
3. V. L. Dotsenko, G. A. Yarovaya, N. A. Golosova, et al., Biokhimiya, 42, 238 (1977).
4. Yu. V. Krizhevskaya, G. A. Yarovaya, G. L. Dotsenko, et al., Biokhimiya, 46, 1510 (1981).
5. E. G. Mirkamalova and I. P. Baskova, Dokl. Akad. Nauk SSSR, 239, 977 (1978).
6. S. Bajaj and K. G. Mann, J. Biol. Chem., 248, 7729 (1973).
7. R. G. Macfarlane, Nature, 202, 498 (1964).
8. S. Magnusson, Ark. Kemo, 23, 285 (1965).
9. T. Morita, S. Ivanaga, and T. Suzuki, Thromb. Res., Suppl. No. 2, 56 (1975).
10. M. Silverberg, J. T. Dunn, L. Garen, et al., J. Biol. Chem., 255, 7281 (1980).
11. L. Svendsen, B. Blomback, M. Blomback, et al., Thromb. Res., 1, 267 (1972).
12. Y. Trauschold and E. Werle, Hoppe Seylers Z. Physiol. Chem., 325, 48 (1961).
13. R. E. Zimmerman, Med. Klin., 73, 145 (1978).

ROLE OF THE MESENCEPHALIC RETICULAR FORMATION IN HORMONE PRODUCTION DURING CHRONIC EMOTIONAL STRESS

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KEY WORDS: stress; bioelectrical activity; hormones; reticular formation.

Investigations of the neurohormonal mechanisms of chronic emotional stress revealed correlation between the functional state of the CNS and hormonal secretion [1, 2]. In continuing the search for neuronal correlates of hormonal secretion, the writers directed their attention to the mesencephalic reticular formation (MRF) which, together with hypothalamic formations, was constantly involved in the activation reaction during stress. It was therefore decided to study the contribution of MRF to the formation of the character of electrical activity of the brain and, in particular, of its hypothalamic formations which are ultimately responsible for hormonal secretion (and, consequently, for supplying the body with hormones under conditions of chronic emotional stress), and also to determine how closely the functional state of the thyroid gland and adrenal cortex is linked with MRF activity under conditions of stress. Information available on this subject is very contradictory and was obtained mainly by acute experiments [3-6, 8, 9, 12-15].

EXPERIMENTAL METHOD

Chronic experiments were carried out on nine cats weighing 3.0-3.5 kg using a model of combined stress caused by immobilization for 4 h accompanied by aperiodic electrodermal stimulation (EDS) of above-threshold strength. The series consisted of four experiments, carried out daily for 1 week. The EEG was recorded on a 17-channel Nihon Kohden (Japan) electroencephalograph by the method described in [1]. MRF was coagulated at coordinates Fr = 2.0, L = 3.5, H = 2.5 of the stereotaxic atlas [7] by a direct current of 1.5-2.0 mA passed for 2 min. The experiments were begun 2-3 weeks after the operation. After the end of the experiments the positions of the recording electrodes were verified histologically. Hormone concentrations were determined by radioimmunochemical assay in blood plasma taken through a

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