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THYMOSIN-α_1, AN ENDOGENOUS MODULATOR OF THE α-THROMBIN RECOGNITION SITE

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Thrombin is the most important bioregulatory enzyme of the homeostasis system and of certain other linked systems [4]. Its physiological functions are largely determined by an additional recognition site of high-molecular-weight substrates, located on the surface of the molecule outside the active center zone [7]. This center is important for the manifestation of specific proteolytic functions and also of the hormonelike properties of α-thrombin during interaction with cells [5, 10]. A substrate locus complementary to the recognition site has been found in the 34-51 region of the Aα-chain of fibrinogen [4] and in the C-terminal fragment 48-65 of hirudin [9]. It can be tentatively suggested that ligands with an amino-acid sequence homologous with these loci will modulate the physiological functions of thrombin. Previously [3] a negatively charged cluster at the C-end of thymosin-α_1, largely homologous with the C-terminal fragment of hirudin, and also a tetrapeptide Arg(Lys)-Glu-Val-Val, homologous with one region of the Aα-chain of fibrinogen, were discovered on the basis of computer screening of regions complementary with the additional recognition site of high-molecular-weight substrates. Thymosin-α_1 inhibited the enzymic activity of α-thrombin relative to fibrinogen and amide and ester substrates. The mechanism of inhibition of activity of this enzyme has not been studied. The investigation described below was undertaken to shed light on this problem.

EXPERIMENTAL METHOD

Bovine α-thrombin was purified from a commercial preparation of USSR/CIS origin by the method in [3]. The clotting activity of the α-thrombin was 2500 NIH U/mg protein, and its esterase activity relative to benzoyl-arginine methyl ester 10 μmoles $\cdot$ min^{-1} $\cdot$ mg^{-1}. β/γ-Thrombin was obtained by restricted proteolysis of α-thrombin by immobilized trypsin [2]. The clotting activity of the β/γ-thrombin did not exceed 7 NIH U/mg protein, and its esterase activity relative to benzoyl-arginine-methyl ester was 9 μmoles $\cdot$ min^{-1} $\cdot$ mg^{-1}. The fibrinogen-clotting activity of α-thrombin was determined turbidimetrically [3]. The amidase activity of α- and β/γ-thrombin relative to H-D-Phe-Pip-Arg paranitroanilide was determined spectrophotometrically in accordance with recommendations of the firm "Kabi Diagnostica." The kinetic constants K_m and V_{max} of hydrolysis of the amide substrate were calculat-

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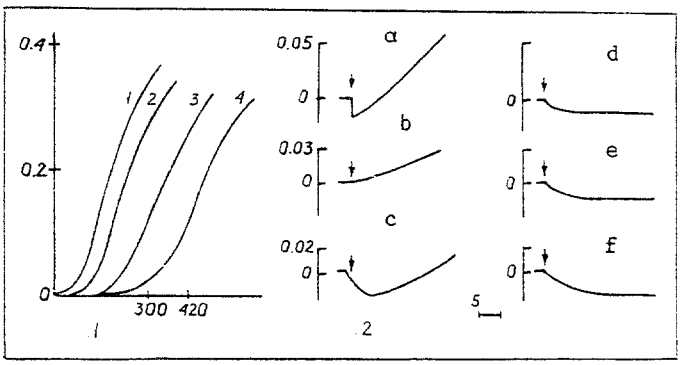

Fig. 1. Effect of thymosin-α_1 on clotting activity of α-thrombin. Curves showing change in light scattering of fibrinogen during fibrin formation under the influence of α-thrombin (1) (10 nM) and in presence of thymosin-α_1; 2) 1.5 nM, 3) 0.015 nM, 4) 0.15 nM. Abscissa indicates reaction time (in sec); ordinate shows intensity of scattering of light at 350 nm (relative units).

Fig. 2. Changes in intracellular pH of peritoneal mast cells in presence of α-thrombin (0.1 nM, a); thymosin-α_1 (0.14 pM, b); α-thrombin (0.1 nM), incubated with thymosin-α_1 (0.14 pM, c); heparin (1 pM, d); α-thrombin (0.1 nM), incubated with heparin (1 nM, e) and with β/γ-thrombin (1 nM, f). Abscissa, reaction time (in min); ordinate, pHi.

ed by an integral method. Changes in the intracellular pH of mast cells isolated from rat peritoneal fluid [12] were recorded with the aid of the fluorescent probe 2,7-bis-carboxyethyl-5/6-carboxyfluorescein (BCECF) by the method in [11]. Commercial preparations of thymosin-α_1 (from "Sigma") and heparin (from "Calbiochem") were used.

EXPERIMENTAL RESULTS

The results of an experiment to study the effect of thymosin on the fibrinogen-clotting activity of α-thrombin are shown in Fig. 1. As is clear from Fig. 1, preliminary incubation of 10 nM thrombin and thymosin within the concentration range from 1 pM to 1 nM led to lengthening of the lag-phase of the polymerization curve (the stage of accumulation of fibrin-monomers), and a decrease in its gradient (the stage of polymerization of fibrin-monomers). The equilibrium constant of inhibition of the clotting activity of thrombin (K_i), calculated between Dixon's coordinates, was 10^{-11} M. Thus our data confirm the effect of inhibition of clotting by the immunoregulator thymosin-α_1 [3]. The absence of total inhibition of enzyme activity of thrombin by thymosin at $K_i = 10^{-11}$ M suggests that thymosin does not bind with the active center of the enzyme, but with an additional recognition site of high-molecular-weight substrates. To shed light on this problem we studied the effect of thymosin on amidase activity of α-thrombin and its β/γ-form with a disturbed structure of the recognition site. The experimental results given in Table 1 show that thymosin, within the concentration range from 1 pM to 1 nM did not alter the kinetics of hydrolysis of α- and β/γ-thrombin. Thrombin evidently does not interact with secondary binding sites located in the enzyme molecule close to the active center, which determine its secondary specificity and are disturbed in the structure of β/γ-thrombin. Binding sites of thymosin are evidently located in domains of the recognition site of high-molecular-weight substrates. Bearing in mind the presence of a large number of negatively charged amino acid residues in the thymosin molecule, it can be tentatively suggested that the complementary site in thrombin will be the anion-

TABLE 1. Kinetic Parameters of Hydrolysis of H-D-Phe-Pip-Arg Paranitroanilide by α- and β/γ-Thrombin in Presence of Thymosin-α_1

Enzyme concentration, 10^{-8} M	Thymosin, M × 10^9	K_m, M × 10^6	V_{max}, M $sec^{-1} \cdot 10^7$	V_{max}/K_m
α-Thrombin	1.5	5.9	5.0	0.08
	0.15	5.3	7.2	0.14
	0.015	6.0	5.0	0.08
	0.0015	4.4	4.4	0.1
β/γ-Thrombin	—	5.8	4.9	0.08
	0.15	4.9	3.1	0.06
	0.015	5.0	2.9	0.06
	0.0015	4.4	3.2	0.07
	—	5.4	3.2	0.06

binding domain of the recognition site. Our observations are in agreement with a published report [6] that the negatively charged C-terminal fragment of hirudin (40-65) inhibits the clotting activity but does not affect the amidase activity of thrombin.

We next studied the action of thymosin on the reaction of α-thrombin with rat peritoneal mast cells. A change in intracellular pH (pH_i), brought about by activity of the Na/H exchanger, is an important mechanism of regulation of the cell response during ligand–receptor interaction on the cell surface. Activation of a whole series of cells by thrombin is known to be accompanied by a change in pH_i [8]. The effect of α-thrombin, its β/γ-form, and of α-thrombin modified by thymosin or heparin, was investigated with the aid of the pH-sensitive probe BCECF. The anticoagulant heparin is known to interact with thrombin through the anion-binding domain of the recognition site of high-molecular-weight substrates [7]. It has been shown that α-thrombin (0.1 nM) causes biphasic changes in pHi: the first phase is a rapid fall of pH_i (by 0.02), the second a subsequent slow rise (by 0.08 in the course of 18 min, Fig. 2a). Modulation of thrombin by thymosin leads to inhibition of the second phase of the pH-response of the cell (Fig. 2c). This is evidently connected with disturbance of thrombin reception due to blockade of the recognition site of high-molecular-weight substrates. In control experiments thymosin (0.14 pM) caused only a very small increase in Na/H-exchange (Fig. 2b). Blocking the anion-binding domain of the recognition site in the thrombin molecule by heparin (1 pM) led to disturbance of transmembrane signal transmission and to total inhibition of the phase of alkalification of mast cell cytoplasm (Fig. 2e). Our results indicate that the thrombin-stimulated change in pH_i of the mast cells is due to the function of the recognition site, interacting with receptors of the plasma membrane. This conclusion was confirmed by experiments in which 0.1 nM β/γ-thrombin, with a disturbed structure of its recognition site (Fig. 2f), was used as inducer of mast cell activation. It will be clear that β/γ-thrombin did not cause alkalification of the cytoplasm, nor did α-thrombin, when modified by modulators of the recognition site of high-molecular-weight substrates.

The results can be summed up by the conclusion that the immunoregulator thymosin-α_1, in very low, near-physiological [3] concentrations, inhibits the functional activity of α-thrombin. This conclusion can be used as an argument in support of the existence of peptide regulation of thrombin in vivo.

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EFFECT OF VITAMINS A, E, C, AND P ON INTENSITY OF EXPERIMENTAL INTRAVASCULAR BLOOD CLOTTING

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Vitamins A and E possess anticlotting, and vitamins C and P a vasoprotective action [2, 12], and they are all antioxidants. By lowering the blood clotting activity of biomembranes they limit thrombin production [10] and reduce the frequency of thrombotic complications in the postoperative period [9].

The aim of this investigation was to study the effect of these vitamins on disturbances of blood clotting and of the microcirculation in experimental exogenous thromboplastinemia.

EXPERIMENTAL METHOD

Experiments were carried out on albino rats (150 ± 15 g), some of which received a mixed diet fortified with vitamins: A) 600 IU, E) 0.15, C) 45, and P) 20 mg/kg body weight daily (for 12 days). On the 13th day the aggregating activity of the platelets, the activated recalcification time (ART), the activated partial thromboplastin time (APTT), the prothrombin index (PI), total antithrombin activity (AA) and antithrombin III (AT-III), fibrinogen activity (FA), concentrations of fibrinogen (FG), PAF, and factor XIII (fXIII), activity of tissue thromboplastin in supramolecular particles of the blood plasma [1, 8], and deformability of the erythrocytes [11] were determined on the 13th day. The rats were then given an injection of a suspension of thromboplastin (0.5 ml/100 g body weight), and blood samples were again taken after 0.5 h. Some of the animals were killed by decapitation and their internal organs removed, fixed in Carnoy's fluid, and embedded in paraffin wax. Sections 5-7 μ thick were stained with Mayer's hematoxylin and eosin. Carbohydrates were revealed by Hale's reaction of colloidal iron binding and the PAS reaction after McManus.

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