literature. It is claimed that experimentally, a specific psychotropic action should be studied only after chronic administration of the preparation, after stabilization of the adaptive changes. It follows from these ideas that the specific activity of lithium hydroxybutyrate ought not to be exhibited until 1 month after its administration, but clinical improvement is known to take place as early as after three daily intravenous injections of the compound in patients with affective mental disorders [1, 6]. The antidepressant action of lithium hydroxybutyrate on a model of reserpine depression is exhibited at about the same time [8]. Lithium hydroxybutyrate is clinically most effective on the 7th-14th days, and the effect does not increase with continued administration [1, 6]. In our experiments it was at these times that the maximal imbalance between synthesis and breakdown of the central 5-HT was observed. Possibly not only stabilization of serotoninergic processes, but also adaptive changes in them may play a definite role in the realization of the psychotropic effect of lithium. It can be tentatively suggested that, by triggering an oscillatory process in the serotoninergic system, lithium itself weakens the pathodynamic structure of the circular psychosis [2], which, in the modern view, is based on fluctuations in the functioning of monoaminergic processes and the balance between them.

LITERATURE CITED

1. G. Ya. Avrutskii, A. A. Neduva, S. N. Mosolov, et al., New Data on the Pharmacology and Clinical Use of Lithium Salts [in Russian], Moscow (1984), pp. 22-30.
2. N. P. Bekhtereva, The Healthy and Sick Human Brain [in Russian], Leningrad (1980).
3. A. V. Val'dman, Neuropharmacology of Antidepressants [in Russian], Moscow (1984), pp. 9-50.
4. B. N. Kogan and N. V. Nechaev, Lab. Delo, No. 5, 346 (1979).
5. M. O. Maimets, "Adaptive changes in dopamine, serotonin, GABA, and benzodiazepine receptors induced by neuroleptics," Author's abstract of dissertation for the degree of Candidate of Medical Sciences, Tartu (1985).
6. S. N. Mosolov, Med. Sestra, No. 4, 36 (1987).
7. D. Yu. Rusakov, "Dynamics of behavior and neurochemical reactivity during chronic administration of antidepressants," Author's abstract of dissertation for the degree of Candidate of Biological Sciences, Moscow (1984).
8. A. S. Saratikov and T. A. Zamoshchina, Zh. Nevropatol. Psikhiat., No. 9, 1362 (1984).
9. A. S. Saratikov, L. L. Fisanova, T. A. Zamoshchina, and S. A. Sakharova, Byull. Éksp. Biol. Med., No. 3, 312 (1986).

ISOLATION AND CHARACTERIZATION OF A PROTEIN C ACTIVATOR FROM *Agkistrodon contortrix contortrix* VENOM

A. N. Storozhilova, M. D. Smirnov,
A. B. Dobrovol'skii, S. V. Kudryavtsev,
and V. N. Titov

UDC 615.919:598.12]:577.152.34].012

KEY WORDS: protein C; activator from snake venom; chromatofocusing; chromogenic substrates

Protein C (PC) is a vitamin K-dependent blood plasma protein which controls the blood coagulation cascade on the negative feedback principle. Thrombin, bound with thrombomodulin on intact areas of endothelium, converts PC into an active serine protease, which degrades factors V and VIII, which localize the blood clotting process [2, 11]. A congenital or acquired lowering of the PC level leads to the development of thrombosis at an early age [1, 3, 11]. There is evidence of a significant fall in the PC level in patients dying within 10 days of

A. L. Myasnikov Institute of Clinical Cardiology, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR, I. K. Shkhvatsabaya [deceased].) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 108, No. 7, pp. 57-59, July, 1989. Original article submitted November 20, 1988.

0007-4888/89/0007-0969$12.50

TABLE 1. Activation of PC by Unfractionated Venoms

Species of snake	Amidolytic activity, A_{405}/min		
	venoms		venom + PC
	chromozyme TH	BCP-300	BCP-300
A. contortrix contortrix	0,278	0,004	0,147
A. halys caraganus	0,072	0,040	0,042
A. blomhoffi ussuriensis A. saxatilis	0,088	0,050	0,060

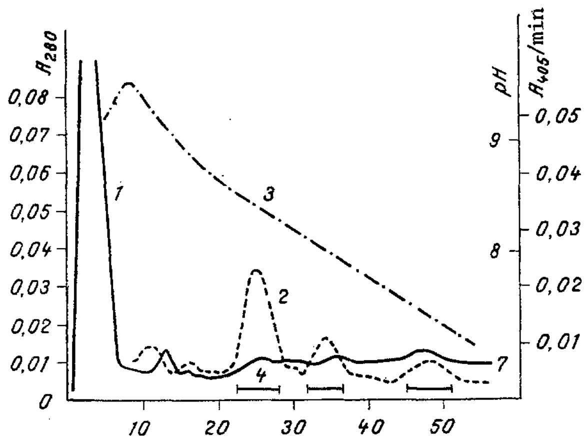

Fig. 1. Chromatofocusing on PBE-94. 150 mg venom applied to column measuring 0.9 × 20 cm at pH 9.5. Eluted with 250 ml polybuffer 96-HCl, pH 7.0. Rate of elution 10 ml/h, volume of fractions 5 ml, absorption at 280 nm; 2) amidolytic activity (A_{405}/min); 3) pH gradient; 4) collected fractions.

myocardial infarction [5]. A connection has been suggested between blood coagulation and hemorrhage after operations on the heart with an artificial circulation and activation of PC [9]. Determination of PC is thus an important diagnostic test in the investigation of pathology of the hemostasis system.

There are two approaches to the determination of PC in plasma: immunologic determination of the protein and determination of activity of PC as a specific protease. The second approach is of great interest because it enables a disturbance of enzyme function in the presence of a normal antigen content to be discovered [13]. PC can be activated in vitro by thrombin after extraction from plasma or from the thrombin–thrombomodulin complex. However, this necessitates subsequent quantitative inhibition of the activator before PC activity can be measured [12]. A protease which activates plasma PC selectively and very rapidly [8] but which does not affect subsequent measurement of its functional activity, has been found in the venoms of two snakes of the *Agkistrodon* genus, living in North America. The aim of this investigation was to study the venoms of snakes of the *Agkistrodon* genus belonging to the fauna of the USSR to discover whether they contain PC activators, and if so, to develop a simple method of obtaining the activator, to enable PC to be determined in plasma by a functional method.

EXPERIMENTAL METHOD

Lyophilized venoms from the snakes *Agkistrodon contortrix contortrix, A. saxatilis, A. blomhoffi ussuriensis,* and *A. halys caraganus* were dissolved in a concentration of 0.1 mg/ml in 0.15 M NaCl containing 1 mg/ml of bovine serum albumin (BSA). To 150 µl of 70 mM HEPES-buffer, pH 8.25, containing 35 mM CsCl, 0.70% polyethylene-glycol 6000, and 1 mM sodium citrate (buffer A) were added 10 µl of PC (32 µg/ml protein) and 10 µl of a solution of the venom for testing. The mixture was incubated for 5 min at 37°C, 30 µl of a 3.3 mM solution of the substrate BCP-300 (supplied by "Behring Diagnostics," West Germany) was added, and A_{405}/min was measured on a Hitachi 150-20 spectrophotometer in a microcuvette with an optical path of 10 mm. The specific amidolytic activity of the venoms was calculated from the value obtained.

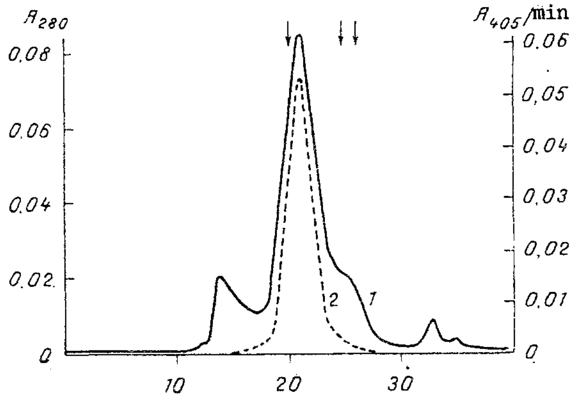

Fig. 2. Gel filtration on Sephadex G-100. Column measuring 1.6 × 80 cm equilibrated with 1% CH_3COOH; protein of fractions 22-28, 32-37, and 45-51 from chromatofocusing column after precipitation with $(NH_4)_2SO_4$ applied to column. Rate of elution 12 ml/h, volume of fractions 5 ml. 1) Absorption at 280 nm; 2) amidolytic activity (A_{405}/min).

A column measuring 0.9 × 20 cm, filled with the ion-exchange resin PBE-94 (from "Pharmacia," Sweden), was equilibrated with 25 mM ethanolamine-acetate buffer, pH 9.5. Venom of *A. contortrix contortrix* (Southern copperhead snake), dissolved in 3 ml and dialyzed overnight against 300 ml of starting buffer, was applied in a dose of 150 mg to the column. The column was washed to remove unabsorbed protein with the starting buffer and eluted with 250 ml of polybuffer 96-HCl, pH 7.0, diluted in the ratio of 1:10. To determine the PC activator, 5 µl of the fraction was added to 0.2 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 0.1 mM of Chromozyme TH ("Boehringer Mannheim," Austria), and A_{405}/min was measured at 37°C. Fractions possessing amidolytic activity were tested for their content of PC activator, using unfractionated venoms.

Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS) was carried out in Laemmli's buffer system [10] in an exponential polyacrylamide gradient from 5 to 20% in gel 0.5 mm thick in a Multiphor II apparatus ("LKB," Sweden). A mixture of standards from 14,400 to 94,000 ("Pharmacia") was used for molecular weight calibration.

EXPERIMENTAL RESULTS

Of four venoms from snakes of the genus *Agkistrodon* investigated, only the venom of *A. contortrix contortrix* induced activation of PC, which was expressed as the appearance of marked amidolytic activity against the chromogenic substrate PCP-300 (Table 1). The unfractionated snake venoms contained a set of proteolytic enzymes capable of degrading PC [4] and lowering its activity. However it is unlikely that this could explain the absence of activity of PC preparations incubated with venoms of related snakes, since Chromozyme TH, which is a good substrate for PC activator [7] is degraded by them 4-7 times less quickly than by the venom of *A. contortrix contortrix*. It was shown previously that PC activator is not present in *A. acutus*, *A. rhodostoma*, and *A. piscivorus piscivorus* [9]. The PC activator has an isoelectric point in the region pH 8.5-7.8 [7]. Since the isoelectric points of most proteins lie within the range pH 7.0-4.0, it might be expected that chromatofocusing in the pH range 9.0-7.0 would be an effective method of purifying the PC activator. As will be clear from Fig. 1, the greater part of the components of the venom, absorbing at a wavelength of 280 nm, is not adsorbed by the carrier at pH 9.5. A polybuffer gradient elutes three fractions at pH 8.35, 7.9, and 7.5, with amidolytic activity against Chromozyme TH, and activating PC. Since the aim of the investigation was to obtain preparative amounts of activator for use in a function test for the presence of PC in plasma, and not the detailed characterization of the isoforms, fractions possessing activator activity were pooled, treated with $(NH_4)_2SO_4$ to 80% saturation, and the residue which formed overnight was collected by centrifugation for 10 min at 15,000 rpm. The protein was dissolved and passed through a Sephadex G-100 column, 1.6 × 80 cm, equilibrated with 1% acetic acid (Fig. 2). The PC activator was eluted from the column in the principal protein peak, which coincided in shape with the peak of activity. This was evidence of the

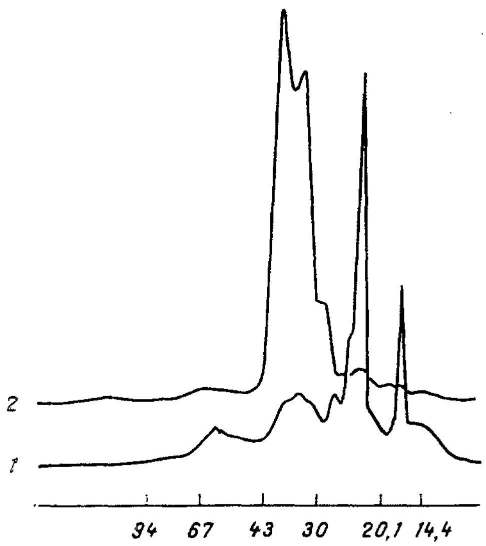

Fig. 3. Densitograms of gels after electrophoresis in polyacrylamide gel gradient in presence of SDS and 2-mercaptoethanol. 1) Original preparation of venom, 2) protein of fractions 19-22. Position of marker proteins and their molecular weight (in kilodaltons) shown along horizontal axis.

high degree of purification achieved in one single stage of chromatofocusing. Fractions 19-22 did not clot a 0.25% solution of fibrinogen in 0.1 M Tris-HCl buffer, pH 7.4, in the course of 30 min.

Electrophoresis in a polyacrylamide gradient in the presence of SDS and 2-mercaptoethanol showed that two proteins with mol. wt. of 37.7 and 31.4 kilodaltons were eluted in this peak (Fig. 3). It is important to note that, unlike the standards forming narrow bands in this electrophoresis system, the test preparation gave crossed diffuse zones, which also are characteristic of the corresponding region of the electrophoretogram of the original venom preparation. Exner and Vaasjoki [4] also obtained a diffuse zone during electrophoresis of activator in a polyacrylamide gel gradient after three chromatographic stages of purification. The most likely explanation of this electrophoretic pattern is the presence of several different isozymes, which can differ both in amino acid composition and in degree of glycosylation. Evidence in support of this hypothesis is given by the presence of three peaks of activity during chromatofocusing (Fig. 1), data in the literature on isoelectric focusing [7], and the character of elution of the enzyme from a concanavalin A-sepharose column [4]. Fractions 19-22 (total volume 20 ml) from the gel-filtration column were pooled and, after addition of 10 mg of BSA ("Sigma," USA), were lyophilized in 1.5-ml aliquots.

To determine PC in plasma, an ampul of the preparation was dissolved in 0.5 ml of 0.15 M NaCl. The main solution of the activator was kept at −35°C. A working solution of activator was prepared by diluting the main solution 1:100 in buffer A. 10 μl of plasma was incubated with 160 μl of activator for 5 min at 37°C, after which 30 μl of a 3.3 mM solution of the substrate BCP-300 was added and A_{405}/min was measured. The amidolytic activity of plasma, measured under similar conditions in buffer A without addition of the activator, and the amidolytic activity of the activator in the absence of plasma were subtracted from this value. Activity in the absence of plasma amounted to 0.010, the same as that for the commercial preparation "Protac" ("Pentapharm," Switzerland), measured under analogous conditions [13]. The relationship between the velocity of the amidolytic reaction and the plasma PC concentration up to 150% of normal is described by the linear equation $A_{405}/\text{min} = -0.0008 + 0.00084 \times [\text{PC\%}]$, with $r^2 = 0.99$.

Thus a preparation which can be used to determine PC in plasma was obtained from the venom of the snake *Agkistrodon contortrix contortrix* by chromatofocusing and gel filtration. Further research will be undertaken to separate its isoforms and to characterize them in detail.

LITERATURE CITED

1. A. W. Brockmans, J. J. Veltkamp, and R. M. Bertina, New Engl. J. Med., 309, 340 (1983).

2. L. H. Clouse and P. C. Comp, New Engl. J. Med., 314, 1298 (1986).
3. B. S. Coller, J. Owen, J. Jesty, et al., Arteriosclerosis, 7, 456 (1987).
4. T. Exner and R. Vaasjoki, Thromb. Haemostas., 59, 40 (1988).
5. R. Conzalez, V. Vicente, A. Alegre, et al., Thromb. Res., 43, 681 (1986).
6. J. H. Griffin, B. Evatt, T. S. Zimmerman, et al., J. Clin, Invest., 68, 1370 (1981).
7. W. Kisiel, S. Kondo, K. J. Smith, et al., J. Biol. Chem., 262, 12607 (1987).
8. J. P. Klein and F. J. Walker, Biochemistry, 25, 4175 (1986).
9. P. N. Knöbl, P. Zilla, R. Fasol, et al., J. Thorac. Cardiovasc. Surg., 94, 600 (1987).
10. U. K. Laemmli, Nature, 227, 680 (1970).
11. R. D. Rosenberg and K. A. Bauer, Hum. Pathol., 18, 253 (1987).
12. N. Sala, W. G. Owen, and D. Collen, Blood, 63, 671 (1984).
13. S. Vigano d'Angelo, P. C. Comp, C. T. Esmon, and A. D'Angelo, J. Clin. Invest., 77, 416 (1986).
14. H. Vinazzer and U. Pangraz, Thromb. Res., 46, 1 (1987).

PREVENTION OF ADRENALIN-INDUCED ARRHYTHMIAS BY THE CALMODULIN BLOCKER TRIFLUOPERAZINE

F. Z. Meerson, I. Yu. Malyshev,
N. P. Larionov, and R. S. Karpov

UDC 616.12-008.318-02:[615.357:577.175.522]-084-092.9

KEY WORDS: arrhythmias; trifluoperazine; isolated heart; adrenalin

Opening of Ca^{++} channels and increased entry of Ca^{++} into the cardiomyocytes are an essential stage in the cardiotonic effect of catecholamines [8], and at the same time they play an important role in the development of adrenergic and, in particular, of stress-induced heart damage [2]. Accordingly blockers of slow Ca^{++} channels, mainly verapamil, diltiazem, etc., have proved to be effective cardioprotective and, in particular, antiarrhythmic drugs [8]. However, this protective effect of Ca^{++}-channel blockers is limited by the fact that after the excess of Ca^{++} has entered the cell or has arisen in the sarcoplasm due to release from the sarcoplasmic reticulum or other depots, damage induced by the excess of Ca^{++} cannot be abolished by blockers of Ca^{++} channels. Accordingly the possibility of preventing the damaging and, in particular, the arrhythmogenic effect of catecholamines by means of blockers of the main Ca^{++} receptor (calmodulin), in the form of a complex with which Ca^{++} can activate phospholipolysis [4], lipolysis [2], proteolysis [6, 12], and peroxidation [1, 10], i.e., processes playing a principal role in the development of adrenergic damage [2], is very interesting.

The aim of this investigation was to study the possibility of preventing depression of the contractile function of the heart and arrhythmias which regularly arise in response to the action of toxic doses of catecholamines, by means of the calmodulin blocker trifluoperazine, and to compare the effect witb the cardioprotective action of the Ca^{++} blocker, verapamil.

EXPERIMENTAL METHOD

Experiments were carried out on male Wistar rats. The rats were heparinized (200 U/100 g body weight, intraperitoneally) and, under pentobarbital anesthesia (50 mg/100 g, intraperitoneally) the heart was removed and placed in a Langendorff perfusion system. Standard Krebs–Henseleit solution (glucose 11 mM) was used for perfusion. The solution was aerated with a mixture of 95% O_2 and 5% CO_2 at 37°C and the pH maintained between 7.3 and 7.4. The perfusion pressure was 9.5 kPa (97 cm water). Mechanical activity of the isolated heart was recorded by Straube's method, using a TD-112S isotonic transducer, and the ECG and mechanical activity of the heart were recorded with the aid of the specialized modules of the RM-6000 polygraph and VC-9 oscilloscope (Nihon Kohden, Japan). One electrode for recording the ECG was placed

Research Institute of General Pathology and Pathological Physiology, Academy of Medical Sciences of the USSR, Moscow. Tomsk Scientific Center, Academy of Medical Sciences of the USSR. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 108, No. 7, pp. 59-62, July, 1989. Original article submitted December 14, 1987.

0007-4888/89/0007-0973$12.50