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THRESHOLD BEHAVIOUR OF THE BLOOD COAGULATION SYSTEM WITH CHANGE IN THE CALCIUM CONCENTRATION*

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The kinetics of coagulation of citrated blood plasma *in vitro* on addition of different calcium concentrations was investigated. It is shown that activation of factor XI does not depend on the calcium concentration, while the thrombin concentration rises non-linearly at a free calcium concentration of 0.2–3 mM. The kinetics of formation of thrombin is well approximated by an exponential relation. The exponent sharply increases in the concentration range of free calcium from 0.2 to 0.5 mM and moves to a plateau at higher concentrations. At free calcium concentrations below 0.2 mM, the thrombin concentration does not rise and coagulation does not occur. The thrombin level is here below the sensitivity of the measuring system (less than 10 pM). Thus, the system behaves in a threshold manner with change in the calcium concentration. The threshold concentration of free calcium is 0.2 mM.

The blood coagulation system possesses a number of unique spatio-dynamic properties, ensuring reliable performance of the vitally important physiological function — protection of the body from the after-effects of damage to the vascular wall. In the zone of damage to the vessel, blood rapidly passes into a new aggregate state — formation of the solid clot. Thus, characteristic of the system as a whole is bistability (trigger behaviour), i.e. it may exist in one of two stable states — fluid or solid.

Outside the blood stream blood rapidly coagulates, i.e. the liquid state of the blood is unstable. The reason may be strong activation of the system after taking blood or the absence of components of the system essential for the liquid state, for example, the vessel walls.

The general dynamics of the coagulation system was analysed and modelled in [1–3]. The main conclusion of these studies was that modern biochemical notions allow one to obtain mathematical models of the system of coagulation possessing threshold behaviour.

The present work seeks to detect experimentally the threshold properties in the coagulation system outside the blood stream. The task is complicated by the fact that after withdrawing the blood there is *in vitro* contact activation of this system, its level being

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always far above the threshold. Therefore, the problem posed cannot be solved by investigating the kinetics of this system *in vitro* at different activation levels.

A way around the problem of the presence of threshold behaviour in the coagulation system is connected with the existence in it of calcium regulation. Accordingly, we experimentally investigated the kinetics of the activation of this system in undiluted fresh donor plasma at different free calcium concentrations. The experiments were run in conditions of constant contact activation. Its value was estimated by measuring the concentration of factor XIa in the system. The kinetics of the formation of a thrombus is best characterized by the thrombin concentration measured in the same experiments. The experiment confirmed the existence in such a system of trigger behaviour as a function of the free calcium level.

Qualitative analysis of the dynamics of the internal and external pathways of the coagulation system was made by Khanin *et al.* [1, 2]. Although the dependence of the kinetics of the process on the calcium concentration was not investigated in these studies, it is not hard to introduce into the data of these models the notion of the role of calcium. From the literature it is known that calcium is an obligatory component of two active complexes which are catalysts of the activation of factor X and II [4]. Thus, to incorporate calcium into the models, it is necessary to postulate that the constants of the corresponding reactions are functions of the calcium concentration.

Perusal of the models shows that the influence of calcium is qualitatively equivalent to the activating effect of the external signals if we consider the response of the system at the thrombin or fibrin level. The activating factor and the rate constants of the reactions depending on the calcium concentration enter the solution in the form of comultipliers of a single generalized activational parameter. This means that for each value of the activational parameter its own calcium threshold concentration exists and vice versa. Since the calcium concentration of the system *in vitro* is readily measured and regulated, this allows one to investigate experimentally the problem of the presence of threshold behaviour in such a system.

MATERIALS AND METHODS

All the experiments were run on thrombocyte-impoverished plasma obtained from the blood of healthy donors and packed into standard plastic containers with "Glyugitsir" preserving citrate solution by centrifugation at 2400 *g* for 20 min at room temperature. Clotting in such a system is due to the fact that a large part of the calcium is bound to citrate. In such plasma because of contact with a foreign surface the contact-activation factors must be activated but the concentrations of the Xa and IIa factors must be very low because of the low concentration of free calcium.

During the experiment the plasma was stored in a sterile plastic container at room temperature. Coagulation was triggered by the addition of $CaCl_2$ solution.

To standardize the content of the coagulation-active phospholipid surface in impoverished plasma "Erilide" — a phospholipid preparation isolated from erythrocyte membranes — was added to all the samples [5].

To measure the concentration of factor XIa, we used the Kabi Diagnostica chromogenic *p*-nitroanilide substrate S2366. The thrombin concentration was measured with the aid of a

specially synthesized substrate (S) BOC-Ala-Pro-Arg-AMC, where BOC is T-N-butoxycarbonyl- and AMC the residue 4-methyl-7-aminocoumarin. A special feature of this substrate is that the product of its splitting (AMC) has a high extinction coefficient which allows one, with its use, to employ spectrophotometry.

The concentration of the free calcium ions in the samples was measured with the Orion ion-selective calcium electrode.

The kinetics of the formation of the active factors IIa and XIa was measured up to the moment of coagulation, by which is understood the time from the moment of addition of calcium to the sample until the fibrin threads wind on to the stirrer.

The fluorimetric measurements of the kinetics of thrombin formation were with the Jobin-Yvon spectrofluorimeter. The reaction product — aminomethylcoumarin — was recorded at the excitation wavelength of 380 nm and emission wavelength of 440 nm. Fluorescence was recorded in the parietal layer of the cuvette, which allows one to reduce sharply the contribution of light scatter of the clot formed in the samples to the measured signal. The controls not containing a fluorogenic substrate showed that with such a regime of recording light scatter makes no measurable contribution to the fluorescence.

The samples contained: Erilide to an end concentration of 0.6 mg/ml, substrate S to an end concentration of 50 μM and different calcium ion concentrations (from 0.15 to 3.4 mM free calcium). All the additives were prepared in buffer containing 0.15 M *Tris*–HCl and 0.1 M NaCl with pH 8.0. The total volume of the additives in all samples was 17% by volume. All the measurements were taken at 37°C with constant agitation.

For the spectrophotometric recording of the kinetics of thrombin formation by optical density, the measurements were made with the Aminco spectrophotometer in the two-wave regime at the wavelengths of 332 nm (isobestic point of the mixture S/AMC) and 340 nm (absorption maximum of the product). Such a method helps to minimize the contribution of light scatter to the measured optical signal. The conditions of measurement and the concentrations of the reagents were similar to those employed in the fluorimetric method of recording.

Factor XIa was measured by spectrophotometry by the two-wave regime with the Aminco spectrophotometer with the chromogenic substrate S2366 in an end concentration 500 μM from the difference in optical densities at the wavelengths 349 and 342 nm (isobestic point of the mixture substrate/*p*-nitroanilide).

With these recording techniques it is possible to measure directly in native plasma where coagulation is triggered with significant change in light scatter in the course of the reaction. This distinguishes to advantage these techniques from most existing ones in which, because of the major contributions of light scatter to the recorded signal, predilution of the system is necessary to avoid possible coagulation.

RESULTS AND DISCUSSION

Figure 1*A* illustrates the dependence of the coagulation time of the plasma (T) on the free calcium concentration in the system. It will be seen that, as the calcium concentration rises, the coagulation time rapidly shortens. As a whole, the curve is similar to a hyperbole as confirmed by Fig. 1*B*, indicating the dependence of the reciprocal of the coagulation time on the free calcium concentration. At a free calcium concentration below 0.19 mM the

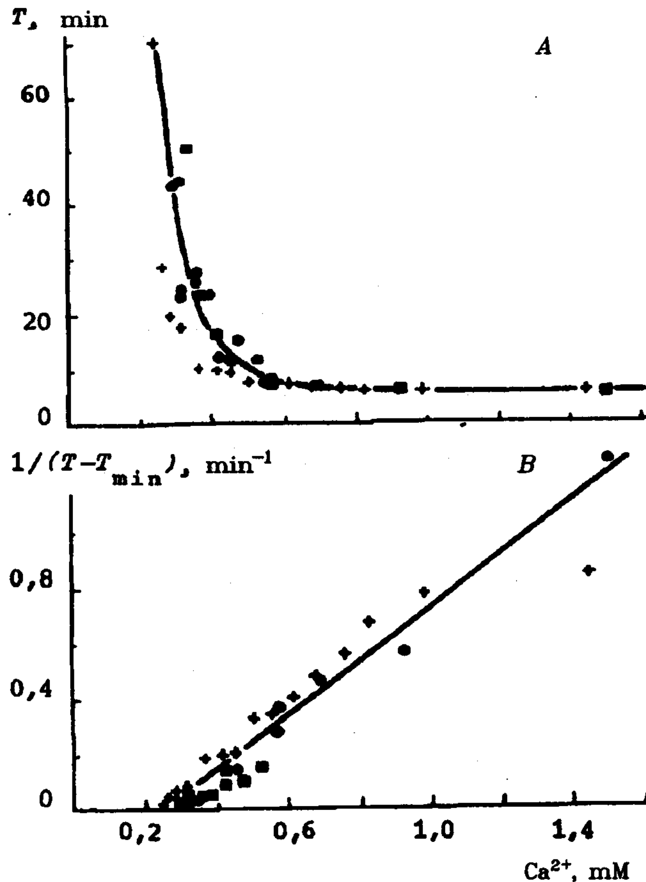

Fig. 1. Dependence of the clotting time of the plasma (T) on the free calcium concentration in the system (A). Dependence of the reciprocal of the coagulation time of the plasma on the free calcium concentration in the system (B). (For more details see the section Results and Discussion.)

coagulation time exceeded the time limits of the experiment (70 min). It seems that at free calcium concentrations well below 0.19 mM coagulation does not occur at all in the system.

Figure 2 indicates the kinetics of splitting of the thrombin substrate S at different calcium concentrations. The rapid rise in the rate of splitting in these curves corresponds to the start of coagulation. It will be seen that the moment of rise in the thrombin concentration closely depends on the calcium concentration.

From the curves presented of hydrolysis of the substrate S is subtracted the background rate of its decomposition in the plasma.

In citrated plasma there is always a certain insignificant background rate of splitting of the substrate S which does not depend on the concentration of calcium added. This rate varies little in different plasmas and does not change in presence of hirudin — a specific thrombin inhibitor which confirms that background splitting of the substrate is due not to thrombin but to other unspecific plasma proteases. This background rate does not exceed 2–3% of the maximum rates of decomposition of the substrate in conditions when coagulation occurs within a time of 30 min.

The kinetics of activation of factor XI in the plasma was studied by measuring the rates of hydrolysis of the chromogenic substrate S 2366 specifically split by factor XIa. Since

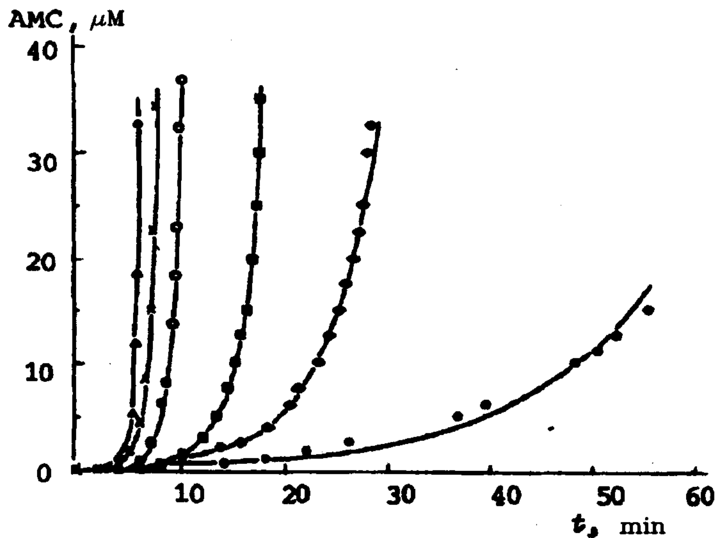

Fig. 2. Kinetics of the accumulation of the product of hydrolysis of substrate S in the same plasma for different free calcium concentrations in the system after subtracting the background. The following free calcium concentrations were used: *1*, 0.24; *2*, 0.26; *3*, 0.31; *4*, 0.36; *5*, 0.50; *6*, 1.44 mM. Results obtained by spectrofluorimetry. Points correspond to experimental data. The lines denote the exponential approximation curves. (For a detailed description of the conditions see Materials and Methods.)

this substrate is quite rapidly split also by the thrombin formed (the rate constant of its hydrolysis on exposure to thrombin is about seven times less than for hydrolysis on exposure to factor XIa) all the measurements with this substrate were made only at the initial stages of the reaction until thrombin begins to make its own contribution to hydrolysis of the substrate.

The walls of the quartz measuring cuvette constantly activate the contact factors of the coagulating system and, therefore, when citrated plasma is placed in the cuvette, splitting of S2366 is immediately observed at rates lying for different plasmas within the range of 0.1–0.2 mM/s. During the experiment the rate of splitting of this substrate slightly decreases. In the plastic cuvette the rate of splitting of S2366 is about half that in quartz and also slightly decreases with time. The picture does not change on addition of different calcium concentrations until the moment when the thrombin formed begins to make its own contribution to the splitting of the substrate, i.e. at the final stages of clot formation.

Figure 3 indicates the dependence of the initial concentration of factor XIa on the free calcium concentration. The kinetics of the formation of factor XIa was investigated with the substrate S2366. The results show that activation of factor XI in the measuring cuvette occurs very rapidly, its level not depending on the calcium concentration in the system but only on the material of the cuvette. The heavy scatter of the measurements of the activity of factor XIa from experiment to experiment appears to be due to different initial activating monitored influences on the plasma which were not monitored. However, they directly determine the activity of factor XIa in each test sample. It was not possible to find any pattern determining this scatter.

The independence of activation of factor XIa from the free calcium concentration suggests that in our experiments on the kinetics of the formation of thrombin in the same

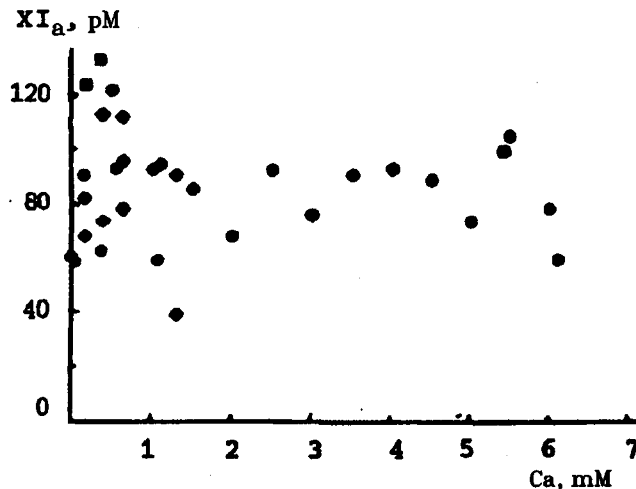

Fig. 3. Dependence of the initial concentrations of factor XIa in the plasma on the free calcium concentration in the system measured by spectrophotometry from the hydrolysis of S2366. The different symbols correspond to different plasma samples. (For the conditions of the measurements see the section Materials and Methods.)

plasma in the presence of different calcium concentrations, the level of the initial contact activation of the internal pathway of the clotting system is identical in all samples.

The kinetics of accumulation of thrombin in the system showed quite different dependence on the free calcium concentration. For the quantitative interpretation of the results, initially the accumulation curves of the products of hydrolysis of substrate S were approximated by an exponential function. The results of this approximation are given in Fig. 2 together with the experimental results. Good agreement is seen with the experiment.

Analysis of the experimentally obtained exponential curves of thrombin formation showed that the exponent ("a") depends on the free calcium concentration in the system. This dependence is depicted in Fig. 4 where it will be seen that there is a certain limiting (threshold) concentration of calcium below which this exponent equals zero. This means

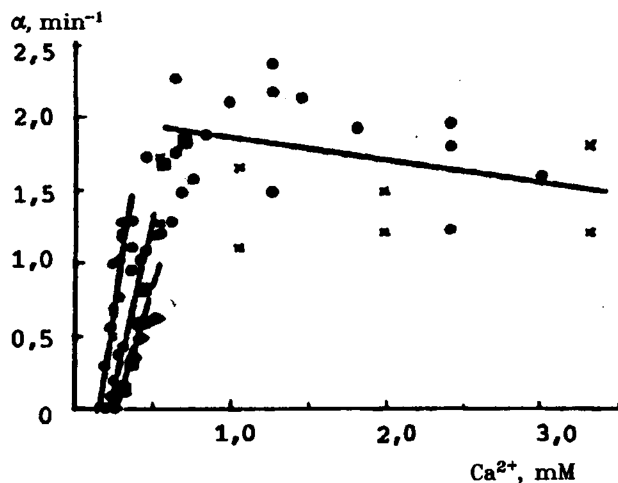

Fig. 4. Dependence of the exponents approximating the kinetics of splitting in the plasma of the substrate ("a") specific for thrombin on the free calcium concentration in the system. (For details see the section Results and Discussion.)

that in this concentration zone there is no rise in thrombin concentration. The threshold free calcium concentration amounts to 0.15–0.24 mM and varies somewhat for different plasmas. With increase in the calcium concentration, the value of the exponent "a" rapidly grows to reach its limiting value at a free calcium concentration above 1 mM. The fairly large value of the scatter in the region of high calcium concentrations is evidently due to rapid increase in the error of the approximation when the initial kinetic curves are very steep.

The conclusion on the presence in the coagulating system *in vitro* of a calcium concentration threshold may also be drawn without approximation of the experimental curves. The tangent to the curve of accumulation of the product in Fig. 2 at the moment of clot formation will give us a value "b" proportional to the thrombin concentration at this moment. The dependence of this value on the free calcium concentration is indicated in Fig. 5. It is easy to see that at calcium concentrations below threshold thrombin does not form in the system.

If we compare the concentrations of the product of splitting of the thrombin substrate at the moment of clot formation (upper points of the curves in Fig. 2), it may be seen that they weakly differ for the different curves, and this is natural since the clot always appears for about the same fibrin concentration but fibrin is the same reaction product for thrombin as AMC. From this it follows that the magnitudes "a" and "b" qualitatively behave identically. This can also be seen from comparison of Figs 4 and 5.

A similar conclusion on the existence in the clotting system of a threshold calcium level also follows from the curve of the dependence of the clotting time on the free calcium concentration (Fig. 1A). In other words this means that an infinitely long clotting time is reached at finite (not zero) calcium concentrations. To satisfy ourselves, we present these results in new coordinates. If this is a hyperbole then the magnitude $1/(T-T_{min})$ must depend linearly on the calcium concentration. This dependence is presented in Fig. 1*B* ($T_{min}=4.5$ min). The experimental points lie fairly well on the straight line intersecting the

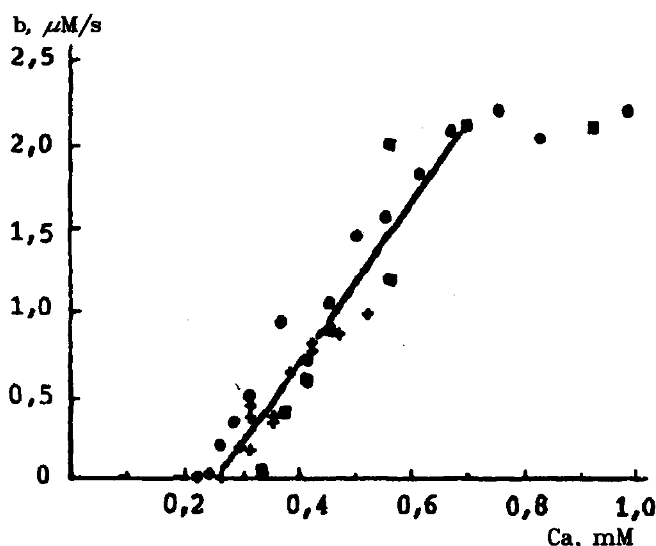

Fig. 5. Dependence of the rate of splitting of substrate S in the plasma at the moment of coagulation ("b") on the free calcium concentration in the system. Data obtained by spectrofluorimetry. The different symbols correspond to different samples of plasma. (For details see the section Results and Discussion.)

abscissa for non-zero calcium concentration. The value of the threshold concentration of free calcium in Fig. 1*B* is 0.2 mM, which is in good agreement with the value obtained from the data on the kinetics of thrombin formation.

The results clearly indicate that *in vitro* the process of plasma clotting depends on the calcium concentration in threshold manner. The threshold calcium concentration apparently must drop with increase in the activation of the clotting system (in our case with increase in the value of contact activation). The precise nature of such a dependence calls for special investigations.

The existence of a calcium threshold is a serious argument in favour of the threshold behaviour of the clotting system in relation to activating factors. The threshold behaviour of the system studied crucially depends on the structure of the feedbacks in this system.

Experimental study of the kinetics of the activation of factors IX and X with change in the calcium concentration may answer the question of which feedbacks play a defining role in the mechanisms of blood coagulation. We plan to investigate the kinetics of activation of these factors in future work.

REFERENCES

1. M. A. Khanin and V. V. Semenov, *Biofizika*, **35**, 139 (1990).
2. M. A. Khanin and V. V. Semenov, *J. Theoret. Biol.*, **136**, 127 (1989).
3. G. M. Willems, T. Lindhout, W. Th. Hermens and H. C. Henker, *Haemostasis*, **21**, 197 (1991).
4. K. G. Mann, M. E. Nesheim, W. R. Church *et al.*, *Blood*, **76**, 1 (1990).
5. A. A. Kozlov, A. L. Barkovskii, V. N. Bovenko and N. Ye. Pichzina, Russian Patent No. 1790604, issued 22 September 1992.