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CALCIUM THRESHOLD IN HUMAN PLASMA CLOTTING KINETICS

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Abstract

In vitro clotting kinetics of citrate human blood plasma under its titration with calcium ions are studied. Contact activation (Factor XIa + plasma kallikrein) is shown to be independent of calcium while non-linear growth of thrombin concentration is demonstrated at calcium concentrations higher than 0.25 mM under conditions of contact activation caused by quartz walls of the measuring cell. Thrombin generation kinetics are well fitted with an exponential function. Power index of the exponential function steeply rises as calcium concentration increases from 0.25 to 0.5 mM and reaches plateau at higher concentrations. At free calcium concentrations under 0.25 mM thrombin level does not grow remaining lower than 30 pM. So, blood coagulation system behaves in a threshold manner under calcium concentration changes. The threshold concentration of free calcium is equal to 0.25 ± 0.05 mM.

Blood coagulation system is characterized by a number of spatio-dynamical features which ensure a reliable protection of an organism from the harmful consequences of vascular wall damage. In the area of vessel injury blood rapidly changes its

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aggregate state from liquid to solid to seal the wound. In other words, the system may exist only in one of two stable states (liquid or solid) and no intermediate semiliquid long-living states are possible. Thus, bistability is an important characteristic of this system as a whole.

Extravascularly blood undergoes rapid clotting suggesting that a liquid state of blood is unstable. This transition may result from activation of coagulation system during blood collection or from a lack of some important components which are responsible for the liquid state of circulating blood (e.g. intact vascular wall).

General dynamics of blood coagulation system were analyzed using mathematical modelling in (1-4). Mathematical models based on the present coagulation data describe threshold behavior of the coagulation system. The threshold behavior implies that blood remains liquid when the concentration of an activator is lower than a threshold level. After overcoming the threshold rapid transition of blood into the solid state occurs.

In the present work we test experimentally whether threshold features are inherent for the blood coagulation system in vitro. Unfortunately, contact activation during the process of blood collection exceeds the threshold level and makes impossible to study dose dependencies of activation kinetics of the coagulation system in vitro. To avoid this problem, we took advantage of the existence of calcium regulation of blood clotting. Examination of the mathematical models of the coagulation system presented below shows that the existence of the activation threshold in all models is strictly coupled to the threshold existing for the dependence of clotting on the ionized calcium concentration. This result is a direct consequence of the consideration of well established biochemical reactions of blood coagulation pathways. We examined the activation kinetics of blood coagulation factor II in fresh donor plasmas at different calcium concentrations and persisting contact activation. The results obtained support the existence of trigger properties of blood coagulation system which are dependent on free calcium concentration.

<u>Mathematical models of calcium regulation in blood coagulation system</u>: Mathematical models of blood clotting were examined in (1-4). Fig.1 shows a diagram of blood coagulation pathways drawn on the basis of the present knowledge.

Qualitative analysis of intrinsic and extrinsic blood coagulation pathways was conducted by Khanin et al.(2,3). In their works the dependence of clotting process kinetics on calcium concentration was not studied in the explicit form, but calcium role may be derived from their models. Calcium is known to be an obligatory component of two active complexes catalyzing factor X and factor II activation (5). In order to include Ca into the models, the constants of the appropriate reactions have to be regarded as functions of calcium concentration (see the schemes in Fig.1).

1. First, we examine the model of the intrinsic pathway without feedbacks (solid lines in Fig.1) as it is presented in (1). The constants $K_2 = k_2 \cdot f(Ca)$ and $K_{10} = k_{10} \cdot g(Ca)$ are

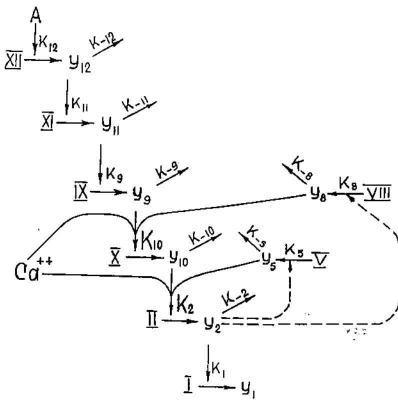

FIG 1.

A scheme of the intrinsic pathway of blood coagulation. Roman numerals correspond to nonactivated factors of the system, yi designate activated form of these factors (or their concentations) ; ki , k-i are the constants of generation and degradation of the corresponding factor. Dashed lines denote the feedbacks.

functions of calcium concentration in this model. The system has a single stationary solution and thrombin concentration is expressed as:

$$y_2(t) = A \cdot f(Ca) \cdot g(Ca) \cdot \left(\prod_i \frac{k_i}{k_{-i}} \cdot S_i\right) \cdot \left(1 - \sum_i E_i \cdot \exp(-k_{-i} \cdot t)\right) \qquad (1)$$

where A is an activation parameter value, S_i are concentrations of the appropriate proenzyme coagulation factors, k_{-i} are inactivation constants of the activated factors and E_i are functions of the constants k_{-i} (i = 12, 11, 9, 10, 2).

It can be easily seen that activating factor value and calcium concentration enter (1) as multiplicands and therefore they are indistinguishable mathematically with respect to their influences on activation kinetics.

This model does not show threshold behavior at all.

2. Inclusion of factor V activation by thrombin (one feedback loop in the intrinsic or extrinsic coagulation pathway) into the model leads to the threshold behavior of the system under activation.

In this model thrombin concentration is expressed as:

$$y_2(t) = \frac{y_2(0)}{(\lambda_1 - \lambda_2)} \cdot \left[(\lambda_1 + k_{-2}) \cdot \exp(\lambda_2 \cdot t) - (\lambda_2 + k_{-2}) \cdot \exp(\lambda_1 \cdot t)\right] +$$

$$+ \frac{y_5(0) \cdot S_2 \cdot k_2 \cdot f(Ca) \cdot g(Ca) \cdot A \cdot G}{\lambda_1 - \lambda_2} \cdot (\exp(\lambda_1 \cdot t) - \exp(\lambda_2 \cdot t)) \qquad (2)$$

where $G = \prod_j \frac{k_j}{k_{-j}} \cdot S_j$, $j = 12, 11, 9, 10$

$$\lambda_{1,2} = -\frac{k_{-2} + k_{-5}}{2} \pm (k_2 \cdot k_5 \cdot f(Ca) \cdot g(Ca) \cdot S_2 \cdot S_5 \cdot A \cdot G + \frac{(k_{-2} + k_{-5})^2}{4})^{\frac{1}{2}}$$

The results of the models' analysis permit us to conclude that in all cases considered calcium effect is essentially equivalent to the activating effects of the external signals when fibrin formation or thrombin generation are considered as the system's response. It is worth mentioning that the threshold calcium concentrations are not the same for the different values of the activation parameters. Since calcium concentration is easily measured and adjusted to the desirable level in the in vitro systems, it is possible to study experimentally whether the threshold behavior does exist.

MATERIALS AND METHODS

All experiments were carried out on platelet poor plasma (PPP) isolated from blood of healthy donors and collected into the standard polyvinylchloride containers with citrate preserving solution "Glugicir" (100 ml of glugicir and 400 ml of blood). To produce PPP, containers with blood were centrifuged at 2400 g for 20 min at 22° C. During the experiments plasma was stored in the sterile polyvinylchloride containers at room temperature.

To standardize the amount of the procoagulant phospholipid surface in PPP, all plasma samples were supplemented with "Erylid", phospholipid fraction of human erythrocyte membranes prepared from outdated packed red blood cells (6). Clotting was initiated by the addition of $CaCl_2$ solution.

Amidolytic assays were carried out with the use of the chromogenic p-nitroanilide peptide S2366 ("Kabi Diagnostica") and a specially synthesized peptide BOC-Ala-Pro-Arg-AMC, where BOC is t-N-butoxycarbonyl and AMC is a residue of 4-methyl-7-amino-cumarine (below we will refer to it as to AMC). The latter peptide is a specific substrate (S) for thrombin (7) and may be utilized not only for spectrofluorometric, but for spectrophotometric determinations of thrombin generation kinetics, too, due to its rather high absorbance coefficient. All other reagents were of reagent grade or of higher quality, and were obtained from commercial sources.

Calcium ion measurements. Calcium ion concentration in PPP was measured with Orion Research ionAnalyzer EA 920.

Fluorescence assay of thrombin formation kinetics. Measurements were performed using Jobin-Ivon fluorescence spectrophotometer JY 3 A. Fluorescence of the reaction product, AMC, was excited at 380 nm and registered at 440 nm.

The sample chamber of the instrument was modified to allow

the fluorescence measurements from the front face of the cuvette in order to minimize the contribution of light scattering during clot formation. Light beam fell on the cuvette wall at the angle of about 40° and fluorescence was measured at 90° angle to the direction of the falling beam. Control measurements performed without the fluorogenic substrate showed no contribution of light scattering into the registered fluorescence. Measurements were carried out with continuous stirring at 37° C.

All samples contained PPP, erylid (final concentration 0.6 mg/ml), the substrate S (final concentration 0.05 mM) and varying concentrations of calcium ions (from 0.15 to 3.4 mM). All additives were prepared using Tris-HCl buffer (0.15 M, pH 8.0) with 0.1 M NaCl. The overall volume of liquid added to the cuvette constituted 17% of plasma volume. AMC concentration was determined from a standard curve constructed for each experiment using dilutions of AMC in plasma.

Spectrophotometric studies on thrombin generation kinetics. Absorbance changes were measured in an Aminco dual-wavelength spectrophotometer using wavelengths 332 nm (isobestic point of the mixture of the substrate S and AMC) and 340 nm (maximal product absorbance). This allowed to decrease markedly a contribution of light scattering into the measured optical signal. Measurement conditions and reagent concentrations were as in the fluorescence assay.

Plasma clotting time measurements. All amidolytic measurements were carried out till the moment of clotting, that is, till the moment when fibrin strands wound on the stirring bar appeared. When the spectrophotometric method was used, a sharp deflection of a recorder pen was seen at this moment due to dramatic increase of noise amplitude resulted from the unavoidable light scattering contribution to the output signal. Fluorescent technique used was virtually free from this disadvantage and in order to notice the moment of clotting we had to discontinue stirring periodically and to determine visually if the stirring bar was clear or coated with fibrin strands.

Amidolytic assay of contact activation. Amidolytic activity of plasma towards the chromogenic peptide S2366 was chosen as a measure of contact activation of plasma under investigation. S2366 can be cleaved by factor XIa , plasma kallikrein, thrombin and activated protein C (8). So, before the two latter enzymes are generated in plasma, amidolytic activity seen with this substrate reflects only factor XIa and kallikrein contribution. The procedure for the estimation of the initial rate of S2366 hydrolysis was essentially the same as that described for thrombin kinetic measurements, except for the substrate and the wavelengths used. S2366 was used at final concentration of 0.5 mM. The isobestic point of the S2366/p-nitroanilide mixture was determined to be 342 nm. The second wavelength, 349 nm, was chosen close to it and far from the maximal product absorbance. The results were expressed as the absorbance changes per second corrected for the own turbidity of each plasma. Correction factor was taken as a ratio of S2366 extinction at 342 nm in plasma to that in the buffer.

RESULTS

A dependence of plasma clotting time on free calcium concentration in the system is presented in Fig.2. As calcium concentration grows, clotting time rapidly decreases. At free calcium concentration lower than 0.19 mM clotting time exceeded the entire duration of the experiment (70 min). Clotting does not seem to occur at calcium concentrations lower than 0.19 mM. The data presented in Fig.2 may be approximated with a hyperbola described by an equation

$$Tcl.= Tcl.min + B/([Ca]-[Ca_{thres}]) \quad (3)$$

where free calcium concentration $[Ca] > [Ca_{thres}]$,

Tcl. is clotting time corresponding to a current calcium concentration [Ca],

$[Ca_{thres}]$ is a minimal (threshold) calcium concentration at which clotting still occurs,
B and Tcl.min are some system parameters. Fitting procedure gave the following estimates of these parameters:

Tcl.min=4.5±0.2 min, B=1.09±0.06 min·mM, Ca_{thres}=0.25±0.05 mM.

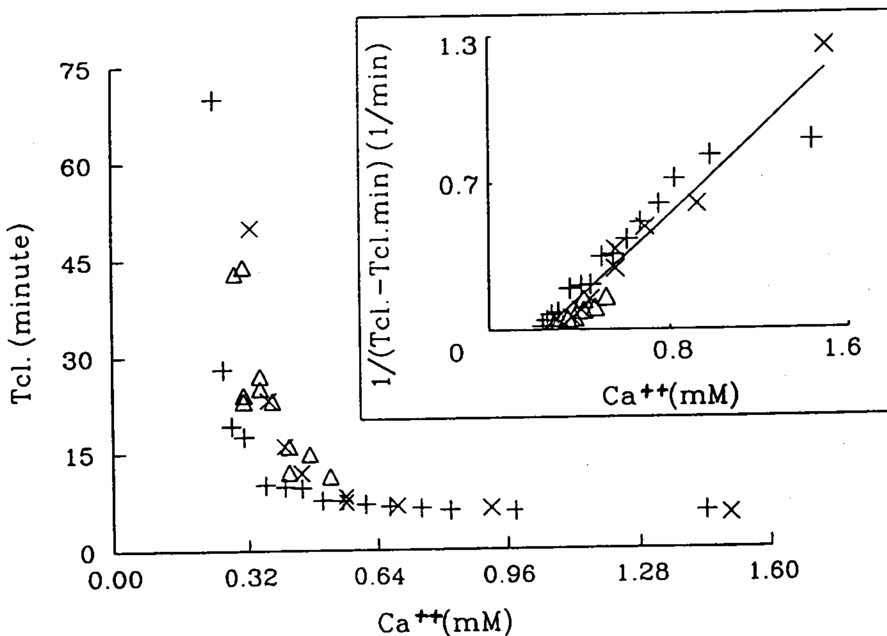

FIG.2.

Dependence of plasma clotting time (Tcl.) on free calcium concentration in the system.
The insert in the right upper corner: Dependence of the 1/(Tcl.-Tcl.min) on free calcium concentration in the system for Tcl.min= 4.5 minutes.
Different symbols correspond to different plasmas. For details see "Materials and methods" and "Discussion".

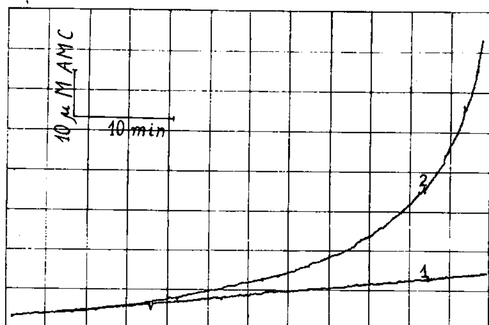

FIG 3.

Typical kinetic curves of the accumulation of AMC, a product of substrate S hydrolysis in plasma, measured fluorimetrically: 1- at free calcium concentration 0.15 mM (the background rate), 2- at 0.29 mM.

The insert to Fig.2 (right upper corner) demonstrates that for Tcl.=4.5 min the reciprocal of (Tcl.-Tcl.min) plotted versus free calcium concentration represents a straight line fairly well.

Plasma clotting time is a variable difficult for quantitative interpretation in the framework of the models described above. Instead, it is more convenient to compare the model and experimental data on coagulation factor activation kinetics. Thrombin generation kinetics were assayed in plasma using fluorescent and spectrophotometric techniques . With both techniques similar results were obtained. Fig.3 shows the time courses of accumulation of AMC, a product of the substrate S hydrolysis, at free calcium concentrations of 0.15 and 0.29 mM.

Generally, small but significant rate of substrate S cleavage is observed in citrate plasma. It does not depend on the concentration of calcium added. If the enzyme responsible for this rate were thrombin its concentration would be about 30 pM (k_2 being 130 sec^{-1} (7)). So we belive that the sensitivity of our methods to thrombin is of the order of 30 pM. This rate does not change in the presence of hirudin, a specific thrombin inhibitor, suggesting that the background substrate cleavage rate is caused by nonspecific plasma proteases rather than by thrombin. Thereafter all substrate hydrolysis rates are presented as differences between measured and background rates.

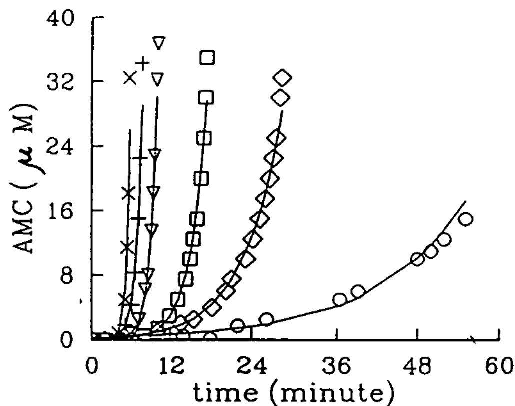

FIG 4.

Kinetics of accumulation of AMC, the product of substrate S hydrolysis, measured fluorimetrically at various calcium concentrations in the same plasma (after the substraction of background rates). (o)- 0.24 mM, (◊)- 0.26 mM, (□)- 0.31 mM, (▽)-0.36 mM, (+) - 0.5 mM, (x)-1.44 mM. Experimental points are approximated with exponential functions using least-square method.

Fig.4 depicts the substrate S cleavage kinetics at different calcium concentrations till the moment of clotting. This moment strongly depends on calcium concentration. At low calcium levels (less than 0.19 mM) substrate S cleavage rate does not grow for the entire period of the observation (see curve 1 in Fig.3).

To interpret quantitatively the results obtained, we approximated the curves of AMC accumulation with an exponential function:

$$y(t)=A\cdot\exp(at) \qquad (4)$$

where y(t) is a concentration of substrate S cleavage product at each moment of time t. The results of this approximation are presented in Fig.4 along with the experimental data. Good coincidence of the approximations and the experiments is seen.

Further analysis of the exponential curves of thrombin generation obtained in experiments has shown that the power index of the exponential function("a" in the equation (4)) depends on calcium concentration in the system. This dependence is depicted in Fig. 5.

The data presented are calculated using results of fluorimetric measurements. Spectrophotometric measurements gave a similar curve for the dependence of "a" on free calcium concentration.

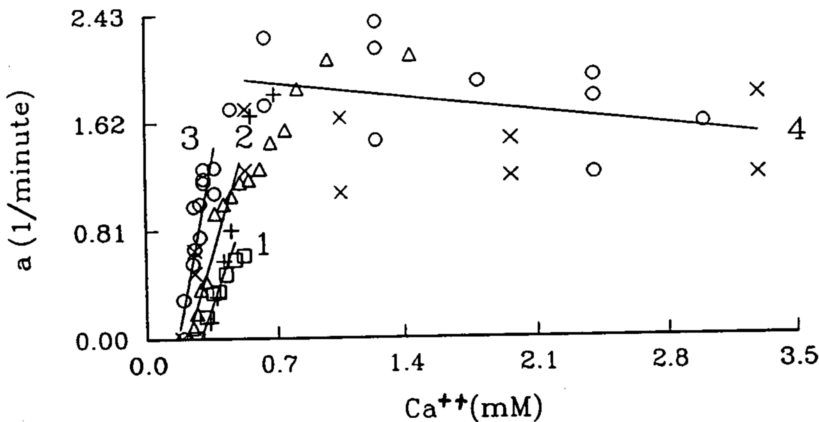

FIG 5.

Power index of exponential functions fitting the kinetics of cleavage in plasma of thrombin specific substrate S as a function of free calcium concentration(for details see "Materials and methods"). Different designations correspond to different plasmas. In the range of low free calcium concentrations regression lines correspond to different plasmas: 1 - to (□,+), 2 - to (Δ), 3 - to (o,x). Line 4 is a regression of the combined data obtained for all plasmas on free calcium concentration in the range of the saturation ion concentrations.

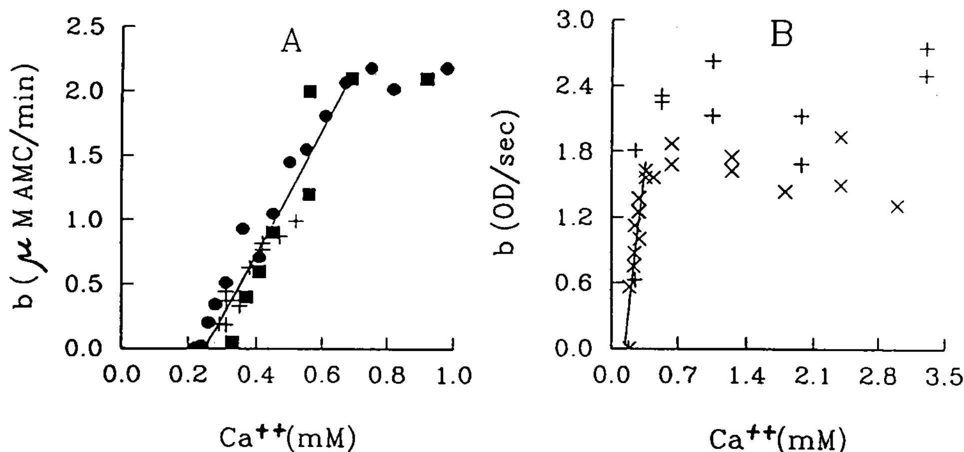

FIG 6.

The substrate S cleavage rate at the moment of plasma clotting as a function of free calcium concentration.
A: Fluorimetric results. B: Spectrophotometric results. Points of different shape correspond to different plasma preparations. In the range of low free calcium concentrations linear regression lines for the experimental data combined for all plasmas investigated are shown. Their equation is $b=c_0+c_1[Ca]$, where [Ca] is free calcium concentration , $c_0 = -1.20 \pm 0.14$ μM/min for A and -1.04 ± 0.25 OD/sec for B, $c_1 = 4.81 \pm 0.32 \cdot 10^{-3}$ min^{-1} for A and 7.64 ± 0.92 $OD \cdot sec^{-1}$ mM^{-1} for B.

An estimation of "b" might be obtained also by using an independent treatment of the experimental data. A tangent to the curve of product accumulation in Fig.4 taken in the point corresponding to the moment of clotting will give us a value of "b" proportional to thrombin concentration at this moment. The dependence of "b" on calcium concentration is shown in Fig.6.

When citrate plasma is placed into a quartz cuvette for the measurements an activation of contact phase coagulation factors occurs and an immediate cleavage of S2366 is observed. The cleavage rates varies from 1 to 3.8 10^{-4} OD/sec for different plasmas. During the experiment the cleavage rate of this substrate slightly decreases as can be seen in Fig.7 (curves 1 and 2).

In plastic cuvettes the cleavage rate of S2366 (curve 2 in Fig.7) is twofold lower than in those from quartz (curve 1). This pattern is not changed when different concentrations of calcium are added (curve 3 in Fig.7 corresponds to calcium concentration of 3 mM). Changes in the substrate hydrolysis rate are seen only when thrombin generated begins to contribute to substrate cleavage, at the late stages of the reaction when clot is forming.

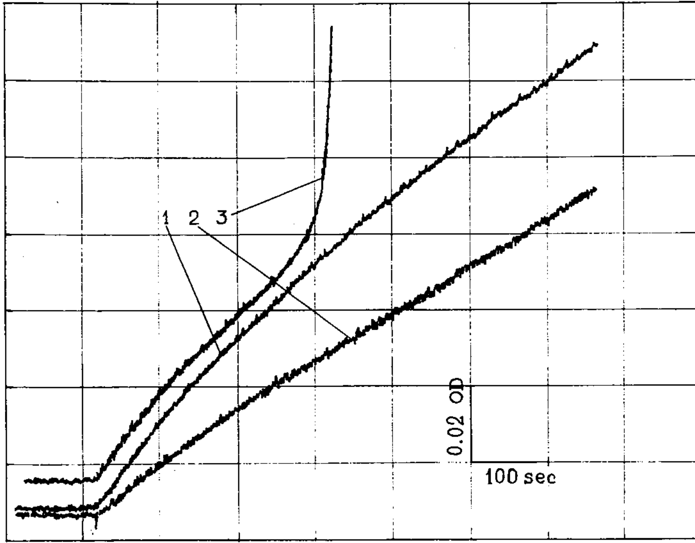

Fig 7.

Typical kinetic curves of substrate S2366 cleavage in the citrate plasma: 1 - quartz cuvette, calcium is not added, 2- plastic cuvette, calcium is not added, 3- quartz cuvette, free calcium concentration is 3mM. (For details see "Materials and methods").

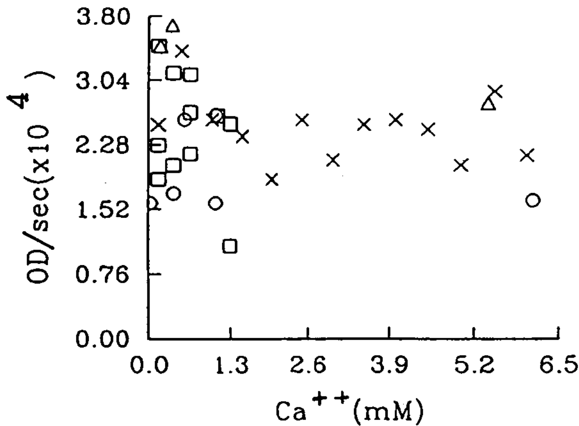

FIG 8.

The initial rate of substrate S2366 hydrolysis in plasma versus free calcium concentration. (For details see "Materials and methods"). Different symbols correspond to different plasma.

The initial rate of substrate S2366 hydrolysis in plasma is insensitive to free calcium concentration in it (Fig.8).

High variability of the results of the amidolytic activity measurements in different plasmas may be related to the different degrees of the initial activation arising during plasma preparation.

DISCUSSION

The results obtained suggest that an activation of factor XI in the cuvette is very rapid. Its level depends on the material of the cuvette rather than on the concentration of calcium

present (see Fig.7). This is in a good agreement with the known data on non-participation of Ca^{2+} in contact activation of blood coagulation system (8).

Independence of the amydolytic activity of plasma from calcium concentration suggests that in our experiments levels of initial contact phase activation of the intrinsic pathway were the same for various concentrations of calcium added.

The kinetics of thrombin accumulation depend on calcium concentration in a somewhat different way. The mathematical models taking into account the feedbacks in the system predict that thrombin generation kinetics are described by exponential function. Good coincidence of the approximation by an exponential function and experimental data is observed. The rate of the function y (expressed by formula 4) variation with time will describe thrombin generation kinetics:

$$z(t) = \frac{1}{k_2} \cdot \frac{dy(t)}{dt} = \frac{A \cdot a \cdot \exp(a \cdot t)}{k_2} = \frac{a \cdot y(t)}{k_2}$$

where z(t) is a current thrombin concentration, k_2 is a kinetic constant for substrate S cleavage by thrombin.

The power index of exponential function ("a") depends on free calcium concentration as it is shown in Fig.5. It is seen that there is a certain limit (threshold) concentration of calcium below which this index is equal to zero. That means that in this concentration range thrombin concentration does not grow. Threshold calcium concentration is about 0.25 mM and slightly varies for different plasmas. As calcium concentration increased, the value of "a", the power index of exponential function, rapidly rose and reached maximum at calcium concentration of 1 mM. Large variability of "a" at high calcium concentrations seems to be related to rapidly growing discrepancy between fitting and original experimental kinetic curves due to their steepness in this range of calcium concentrations.

A conclusion on the existence of the threshold calcium concentration should exceed to trigger the system in vitro might be done not only by fitting the experimental curves. A dependence of the parameter "b" proportional to thrombin concentration at the moment of clotting on free calcium concentration shown in Fig.6 suggests that thrombin is not formed in the system when calcium concentration is below the threshold.

Analogous conclusion on calcium threshold existence in the coagulation system might be also extracted from the plot of clotting time as a function of free calcium concentration (Fig.2). Experimental points lie on a straight line intersecting abscissa axis at non-zero calcium concentration value. The threshold calcium concentration value obtained in such a way from the data presented in Fig.2 is equal to 0.25 mM, in a good agreement with the threshold value determined from kinetic data of thrombin generation.

The data obtained clearly suggest that blood coagulation process depends on calcium concentration in a threshold manner. It seems reasonable to suggest that the threshold calcium concentration would be lower for activated plasmas depending on the extent of their activation (contact activation in our case) than for non-activated. However, special investigations are necessary to understand the true character of such a dependence.

Calcium threshold existence strongly supports the hypothesis that activating factors also have to exceed the threshold to trigger coagulation process. The threshold behavior of the system under consideration depends mainly on the way in which the feedbacks are organized in it.

Experimental study of calcium effect on factor IX (and/or X) activation kinetics might clarify the question what are the feedbacks playing principal roles in blood coagulation mechanisms.
We suppose to investigate the activation kinetics of these factors in our future work.

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