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Non-proteolytic activation of cellular protransglutaminase (placenta macrophage Factor XIII)

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Plasma Factor XIII is a zymogen (plasma protransglutaminase) with the tetrametric structure A_2B_2, whereas the cellular protransglutaminase, i.e. Factor XIII in the platelet and monocyte/macrophage, consists exclusively of A subunits (A_2). It is generally accepted that at Ca^{2+} concentrations comparable with that in plasma the proteolytic removal of an *N*-terminal activation peptide is the prerequisite for the Ca^{2+}-induced formation of a catalytically active configuration of subunit A. In this study it was demonstrated that at high concentrations NaCl or KCl induced a non-proteolytic activation of cellular (placental macrophage) but not plasma protransglutaminase. The activation depended on time and salt concentration, and Ca^{2+}, in the range 0–20 mM, greatly enhanced the activation process. At 1.25 M-NaCl maximal activation occurred within 60 min in the presence of 2 mM-$CaCl_2$, and even at physiological NaCl concentration a slow progressive activation could be observed in the presence of Ca^{2+}. The specific activity of salt-activated Factor XIII was 1.5–2.0-fold higher than that obtained after thrombin activation. The non-proteolytic activation of cellular protransglutaminase was abolished by the addition of subunit B of plasma Factor XIII in stoichiometric amount, which suggests that (one of) the physiological function(s) of the B subunit in plasma Factor XIII is to prevent the slow spontaneous activation of A subunit that would occur in a plasmatic environment.

INTRODUCTION

Factor XIII (FXIII) of blood coagulation present in the plasma is a zymogen (plasma protransglutaminase) of tetrameric structure (A_2B_2). The active site formed in the course of an activation process is located on the A subunit while subunit B remains enzymically inactive. The presence of FXIII has also been verified in platelets [1], monocytes and monocyte-derived macrophages [2–7]. In contrast with the plasma FXIII, however, this cellular protransglutaminase consists exclusively of A subunits (A_2). The active transglutaminase (FXIIIa) formed from FXIII catalyses an acyl-transfer reaction in which the carboxamide group of a peptide-bound glutamine residue is the acyl donor and the primary amino group of a low-M_r amine or a peptide-bound lysine residue is the acyl acceptor. In the latter case an ϵ-(γ-glutamyl)lysyl bond is formed and as a result two peptides chains become covalently cross-linked (for reviews see [8–10]). The main physiological function of plasma transglutaminase is to cross-link fibrin chains and to bind α_2-antiplasmin to fibrin in the terminal phase of blood clotting and by this means ensure clot stability. The physiological function of cellular FXIII has not been elucidated.

It is generally accepted that the first step in the physiological pathway of FXIII activation is the proteolytic removal of an activation peptide from the *N*-terminal end of subunit A, which is followed by Ca^{2+}-induced changes in the conformation of this subunit. As a result the B subunits dissociate (in the case of plasma FXIII) and the A subunits assume the active configuration. The proteolytic enzyme physiologically involved in the activation of plasma FXIII is thrombin. Among the intracellular proteinases calpain [11] and cathepsin C [12] have been shown to be able to activate platelet FXIII, although in physiological conditions their involvement in the activation of cellular FXIII still remains to be established. The release of activation peptide, however, does not seem to be an absolute requirement for the activation of zymogen. It has been demonstrated that at non-physiologically high concentration ($\geq$ 0.1 M) Ca^{2+} induces activation of plasma FXIII in the absence of any proteolysis [13–15]. In the present study we show that NaCl or KCl induces a concentration- and time-dependent activation of intact cellular but not plasma FXIII at Ca^{2+} concentrations comparable with those required for the activation of thrombin-treated FXIII. The addition of subunit B prevents this proteolysis-independent activation process.

MATERIALS AND METHODS

Glutamate dehydrogenase, human thrombin (3000 NIH units/mg of protein), alkaline phosphatase (type VII-S), ADP, NADPH, dithiothreitol, Hepes and putrescine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), DEAE-Sepharose CL-6B and Sephacryl S-300 were from Pharmacia (Uppsala, Sweden), ethylamine hydrochloride was from Fluka (Buchs, Switzerland), Hammarsten casein was from Merck (Darmstadt, Germany), and M_r standard proteins and [1,4-^{3}H]putrescine (18.6 Ci/mmol) were from Amersham International (Amersham, Bucks., U.K.). Affi-Gel 501 (organomercurial–agarose) and chemicals for SDS/PAGE were the products of Bio-Rad Laboratories (Richmond, CA, U.S.A.). All other chemicals were of reagent grade. Acetylated dephosphorylated β-casein was prepared in our laboratory [16].

The cellular protransglutaminase used in the experiments was placenta FXIII, which is located intracellularly in placental macrophages [7]. Fibrogammin (Behringwerke, Marburg, Germany), a commercially available human placenta FXIII (A_2) preparation, was kindly given by Dr. H. Karges. It was further purified to obtain an electrophoretically homogeneous protein preparation. The contents of one bottle of Fibrogammin HS (370–685 mg of solid) were dissolved in 30 ml of 50 mM-Tris/HCl

Abbreviations used: FXIII, Factor XIII of blood coagulation; FXIIIa, activated FXIII (EC 2.3.2.13).

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buffer, pH 7.5, containing 1 mM-EDTA (buffer A). FXIII was separated by ion-exchange chromatography on a DEAE-Sepharose CL-6B column (5 cm × 18 cm) with a linear 0–0.3 M-NaCl gradient in buffer A. The individual fractions were tested for FXIII activity by the u.v. kinetic method [17]. To the pooled FXIII-containing fractions was added $(NH_4)_2SO_4$ to 40% saturation and the pH was adjusted to 5.4 with 1 M-acetic acid. The resulting precipitate was pelleted by centrifugation, dissolved in a small volume (2–2.5 ml) of buffer A and gel-filtered on a Sephacryl S-300 column (1.6 cm × 60 cm). Fractions containing electrophoretically homogeneous FXIII subunit A were freeze-dried in the presence of 5% (w/v) sucrose and stored at −20 °C. Before use the reconstituted material was dialysed against buffer A and its protein concentration was determined. The specific activity of thrombin-activated FXIII as measured by the u.v. kinetic assay was 13.2±2.4 nkat/mg ($n = 4$).

Plasma FXIII (A_2B_2) was purified from outdated human plasma by the method of Lorand *et al.* [14]. Subunit B of plasma FXIII was isolated by the method of Schwartz *et al.* [18]. Trace amounts of A subunit contaminating the preparation were removed by affinity chromatography on Affi-Gel 501 [19].

Purified placenta or plasma FXIII was activated at 37 °C by thrombin for 10 min or, alternatively, by NaCl or KCl for the time periods indicated. The activation mixture contained 70 μg of placenta FXIII/ml, 50 mM-Tris/HCl buffer, pH 7.5, 9 mM-dithiothreitol and various concentrations of $CaCl_2$, NaCl or KCl. The concentration of thrombin (if present) was 40 units/ml. In blanks 5 mM-EDTA replaced the $CaCl_2$.

Transglutaminase activity was measured by the u.v. kinetic method [17] or by the [^{3}H]putrescine-incorporation filter-paper assay [20] at 37 °C. The following components of the assay mixtures were identical for both methods: 5.6 μg of placenta FXIII/ml or 11.2 μg of plasma FXIII/ml, 70 mM-Hepes/NaOH buffer, pH 7.5, 1.7 mM-dithiothreitol, 0.1 M-NaCl or -KCl (1 M-NaCl in certain cases) and 10 mM-$CaCl_2$ or 5 mM-EDTA (in the blank). In the case of the u.v. kinetic method the above assay mixture was supplemented with 20 g of acetylated dephosphorylated β-casein/l, 35 mM-ethylamine, 0.6 mM-ADP, 6 mM-2-oxoglutarate, 20 kunits of glutamate dehydrogenase/l and 0.32 mM-NADPH, whereas [^{3}H]putrescine-incorporation method was carried out with 2 g of Hammarsten casein/l and 0.45 mM-[^{3}H]putrescine (11.1 mCi/mmol; a 1:10 dilution of the stock [^{3}H]putrescine solution with 10 mM unlabelled putrescine) substrates. The u.v. kinetic assay was performed on an Encore centrifugal fast analyser (Baker Instruments, Allentown, PA, U.S.A.).

Placenta FXIII activated with thrombin (40 units/ml for 10 min) or NaCl (1 M-NaCl for 30 min) as well as non-activated FXIII were denatured in reducing conditions. A 9 μg portion of denatured FXIII was analysed by SDS/PAGE in 7.5% polyacrylamide gel by the procedure of Weber & Osborn [21]. Densitometric scanning of Coomassie Blue-stained gels was carried out on a Cliniscan densitometer (Helena Laboratories, Beaumont, TX, U.S.A.). The dansyl method was used for *N*-terminal amino acid determination [22]. The concentrations of purified plasma FXIII, placenta FXIII and FXIII subunit B solutions were determined both by using the Pierce (Rockford, IL, U.S.A.) Protein Assay Kit and at 280 nm by using the absorption coefficient $A^{1\%}_{1\,cm,280}$ 13.8 for calculation [18]. The two methods gave essentially identical results.

RESULTS AND DISCUSSION

When incubated in the presence of 1 M-NaCl a time-dependent activation of placenta macrophage FXIII occurred (Fig. 1). This phenomenon could be demonstrated equally well by two different

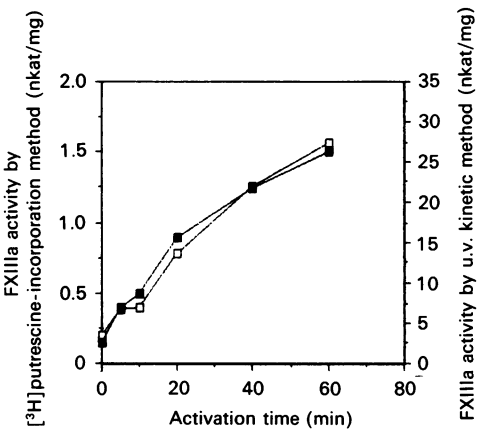

Fig. 1. Time-dependent activation of placenta macrophage FXIII in the presence of 1 M-NaCl

FXIIIa (transglutaminase) activity was determined by the u.v. kinetic (■) and [^{3}H]putrescine-incorporation (□) methods. Activation occurred in the presence of 2 mM-$CaCl_2$. Conditions for the assay are given in the Materials and methods section. These experiments were performed three times with essentially identical results.

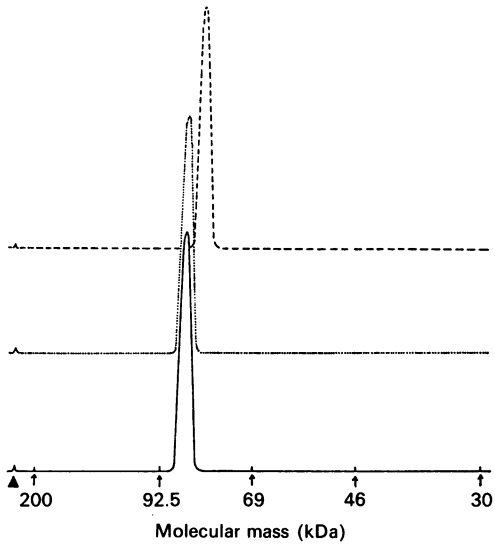

Fig. 2. Lack of release of activation peptide during 1 M-NaCl-induced activation of placenta FXIII

Non-activated (——), thrombin-activated (------) and 1 M-NaCl-activated (·····) placenta FXIII were subjected to SDS/PAGE. The densitometric tracings of Coomassie Blue-stained gels are shown. ▲ indicates the top of gels. Arrows indicate the positions of respective M_r standard proteins.

assay systems. The u.v. kinetic assay monitors the amount of ammonia released from peptide-bound glutamine residues in the first step of the enzyme reaction [17], and the [^{3}H]putrescine-incorporation method measures the amount of primary amine covalently bound to the glutamine-donor protein substrate through an isopeptide bond [20]. Though specific activities obtained with the two methods differed considerably, the relative rates of activation were identical. It is to be emphasized that the activation induced by 1 M-NaCl occurred at 2 mM-$CaCl_2$ concentration, a value close to the physiological plasma Ca^{2+} concentration.

As in the above experiments the concentration of NaCl

changed drastically when samples from the activation mixture were transferred into the assay systems (from 1 M to 0.1 M), and there was a possibility that the NaCl-induced activation was partially reversed during activity measurement and that in this way the extent of activation was underestimated. The following results indicate that this did not occur to a significant extent. (1) By both methods constant transglutaminase activities were measured until substrate exhaustion occurred. (2) In the case of thrombin-activated placenta FXIII, where the removal of activation peptide is clearly irreversible, the ratio of transglutaminase activities measured at 0.1 M- and 1 M-NaCl concentrations was 1.7 ± 0.2 ($n = 4$). When the same experiment was carried out with 1 M-NaCl-activated enzyme, ratios comparable (1.4 ± 0.2; $n = 4$) with the above values were obtained.

It was important to exclude the possibility that the activation that we observed was due to an unidentified proteolytic enzyme active only at high NaCl concentration and present in FXIII preparations in an amount undetectable on SDS/PAGE. As demonstrated by SDS/PAGE, the mobility of NaCl-activated enzyme was distinctly different from that of the thrombin-activated one, and it co-migrated with non-activated FXIII (Fig. 2). In addition, no free *N*-terminal amino acid could be detected in FXIII activated by 1 M-NaCl (the *N*-terminus is blocked in the intact molecule), i.e no proteolytic cleavage of zymogen took place during the activation process.

Fig. 3 demonstrates the dependence of placenta FXIII activation on NaCl concentration. When KCl replaced the NaCl, activation to the same extent occurred (not shown in the Figure). It may be noted that the maximal specific activity induced by NaCl or KCl was 1.5–2.0-fold higher (in five different experiments) than that obtained with the thrombin-activated enzyme.

The expression of transglutaminase activity induced by NaCl or KCl required the presence of Ca^{2+} during activity measurement. When Ca^{2+} was removed by EDTA no activity could be detected. The activation process itself also showed a clear dependence on Ca^{2+} concentration (Fig. 4). When transglutaminase activity was induced by 1 M-NaCl a saturating concentration for $CaCl_2$ was reached at around 20 mM, and even at 1 mM 60% of the maximal activity could be obtained. The observed dependence of 1 M-NaCl-induced activation of placenta FXIII on Ca^{2+} concentration is quantitatively well comparable with that of thrombin activation of platelet FXIII [23]. Unexpectedly, preincubation of placenta FXIII with 1 M-NaCl at zero $CaCl_2$ concentration, irrespective of whether that meant no added $CaCl_2$ or the removal of traces of Ca^{2+} by EDTA, induced

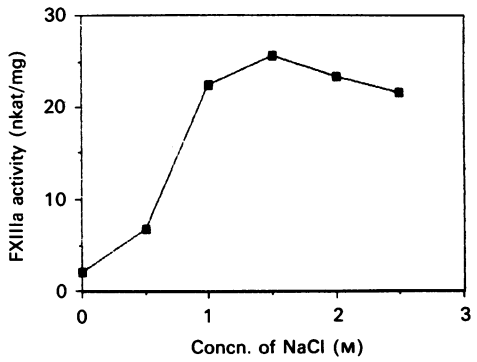

Fig. 3. NaCl-induced activation of placenta macrophage FXIII: dependence on NaCl concentration

Activation mixtures containing 2 mM-$CaCl_2$ were incubated for 60 min. Other assay conditions are given in the Materials and methods section.

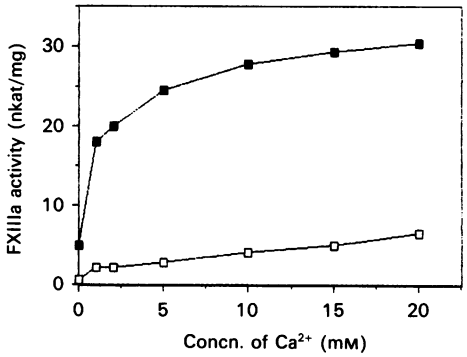

Fig. 4. Ca^{2+}-dependence of the activation of placental macrophage FXIII induced by NaCl

The activation mixtures contained 1 M-NaCl (■) or 0.15 M-NaCl (□). FXIIIa (transglutaminase) activity was determined after a 30 min period of activation.

a low but reproducible transglutaminase activity. This result suggests that at 1 M-NaCl even without Ca^{2+} a kind of potential active site is preformed that, under assay conditions, results in the expression of a low transglutaminase activity. The finding that thrombin-treated platelet FXIII reacts with an active-site reagent in the absence of Ca^{2+} [24] also leads to the conclusion that, if subunit B is absent, Ca^{2+} is not an absolute requirement for the formation of a potentially active configuration. Ca^{2+}, however, either greatly facilitates the activation process or helps to bring about a configuration of a much higher catalytic activity. At 0.15 M-NaCl no activation could be detected without Ca^{2+}. By increasing the Ca^{2+} concentration a gradually increasing activation of FXIII was observed but, as compared with activities obtained with 1 M-NaCl, the extent of FXIII activation induced at physiological NaCl concentration was rather insignificant (by one magnitude lower). It is to be noted, however, that even at 1 mM-$CaCl_2$ a slight activation was detectable, and in this case 20 mM-$CaCl_2$ was not sufficient to saturate the reaction.

The above experiments clearly demonstrate that in the presence of 1 M-NaCl at relatively low Ca^{2+} concentration the dimeric cellular FXIII can assume an enzymically active configuration without the proteolytic removal of the activation peptide. As mentioned, a proteolysis-independent activation of the tetrameric plasma protransglutaminase could be demonstrated only at very high Ca^{2+} concentrations ($\geqslant 0.1$ M), although the Ca^{2+} requirement was somewhat decreased when high concentrations of strongly chaotropic anions, such as KSCN, were present [13–15]. The salt-induced activation that we observed with the cellular zymogen required about 50-fold less Ca^{2+}. Under conditions in which full activation of cellular FXIII occurred plasma FXIII remained virtually inactive (Fig. 5).

The mechanism of intracellular activation of cellular FXIII is not known. The Ca^{2+}-sensitive proteinase calpain has been proposed as proteolytic activator [11], but its intracellular involvement in such a process has never been proved. It is noteworthy that at acidic pH FXIII was activated in platelet lysate and by a commercial preparation of cathepsin C, and it was concluded that platelet FXIII may be activated by endogenous acid proteinase(s) [12]. However, the unchanged M_r of FXIII and the lack of any new *N*-terminal amino acid following activation [12] clearly rule out the involvement of any proteolytic action in those experiments. In the light of our results it seems possible that under the assay conditions used in the above experiments the intact zymogen became activated. The physiological significance of our finding is not clear as yet. A local

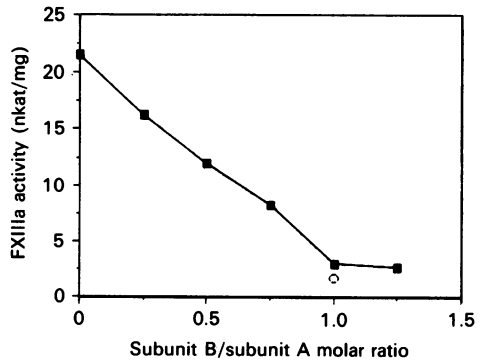

Fig. 5. Inhibitory effect of FXIII subunit B on the 1 M-NaCl-induced activation of placenta FXIII

Activation mixtures (see the Materials and methods section) containing 2 mM-$CaCl_2$ were supplemented with different amounts of isolated B subunit of plasma FXIII to give subunit B/subunit A molar ratios as indicated on the abscissa. The mixtures were tested for transglutaminase activity after a 30 min activation period. For comparison an equivalent concentration of plasma FXIII (A_2B_2) subjected to the same procedure was also tested for transglutaminase activity (○).

intracellular rise in ionic strength to such an extent is hardly probable, although a slow activation by a moderate local increase in neutral salt concentration, especially when platelets become permeable to plasma Ca^{2+} in the clot, cannot be ruled out. The fact that high ionic strength induces the formation of enzymically active zymogen raises the question whether the same active configuration could be brought about by other means, e.g. by interaction with certain cellular components. Clearly, further experiments are needed to investigate such speculations.

The finding that non-proteolytic activation of FXIII at high NaCl and KCl concentrations occurred only with the dimeric (A_2) but not with the tetrameric (A_2B_2) proenzyme strongly suggested that in the latter case the presence of B subunits prevented the native A subunits from assuming an active conformation or that the B subunit abolished the stabilization of such an enzymically active configuration. To test this hypothesis various amounts of B subunit were added to a fixed concentration of A subunit, and after preincubation with 1 M-NaCl the transglutaminase activities were measured. Fig. 5 shows that the activation of FXIII gradually decreased as the molar ratio of B subunit to A subunit increased. On reaching equimolar concentrations the salt-induced activation became abolished. In separate experiments it was shown that the addition of B subunit did not inhibit the enzyme that had been activated by NaCl in its absence, i.e. the B subunit exerted its inhibitory effect on the process of activation. Results shown in Fig. 3 suggested that in the absence of B subunit a slow non-proteolytic activation of subunit A takes place even at NaCl and Ca^{2+} concentrations present in a plasmatic environment. Further experiments clearly showed that a slow progressive activation of A subunit occurred at 0.15 M-NaCl and 2 mM-$CaCl_2$ concentrations, reaching a transglutaminase activity of 6.5 nkat/mg within 6 h. This slow progressive activation was also prevented by the addition of an equimolar amount of B subunit.

The physiological function of subunit B in plasma FXIII has not been elucidated. It is rather difficult to envision a role for an 'inhibitory' subunit associated with the anyway inactive zymogenic A chain. The involvement of subunit B in a process functionally unrelated to fibrin stabilization, namely in the inhibition of contact activation at the late stage of blood coagulation, has been proposed [25]. It might also be of importance in protecting the A chain from proteolytic inactivation by thrombin [26]. Our present study suggests that under plasmatic conditions the A chains, without associated B subunits, would not stay inactive but go through a slow progressive activation and that the physiological function or one of the physiological functions of subunit B is to prevent that slow proteolysis-independent activation process.

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