Magnesium(II) Is a Crucial Constituent of the Blood Coagulation Cascade

POTENTIATION OF COAGULANT ACTIVITIES OF FACTOR IX BY Mg$^{2+}$ IONS

We recently showed that not only Ca$^{2+}$ ions but also Mg$^{2+}$ ions play a crucial role in stabilizing the native conformation of coagulation factor IX. We here report that Mg$^{2+}$ ions at physiological concentrations greatly augment the biological activities of factor IX. In clotting assays with dialyzed plasma, addition of Mg$^{2+}$ ions enhanced the apparent coagulant activity of factor IXa, while that of factor Xa was scarcely affected. Activation of factor X by factor IXa in the presence of factor VIIIa, phospholipids, and Ca$^{2+}$ ions was accelerated by Mg$^{2+}$ ions. It appeared that the cation increased the affinity between factor IXa and factor VIIIa, thereby increasing the apparent catalytic efficacy of the enzyme. We also evaluated the effect of Mg$^{2+}$ ions in the coagulation pathway initiated by tissue factor and found that activation of factor IX by factor VIIa-tissue factor was accelerated by the cation. Consequently, clotting of normal plasma induced by factor VIIa-tissue factor was shortened by the cation, while no such effect was observed in plasma deficient in factor IX or VII. These results indicate that the previously unrecognized plasma component, Mg$^{2+}$ ions, plays crucial roles in blood coagulation and, moreover, that contributions of factors IX and VIII in the coagulation cascade have been seriously underestimated in previous investigations.

The coagulation proenzyme factor IX plays a central role in hemostasis in concert with its essential procofactor factor VIII, and is apparent from the fact that genetic defects in these factors cause a life-threatening tendency to bleed, hemophilia (1). Once activated, factor IX forms a complex with the activated form of factor VIII (IXa-VIIIa)1 on phospholipid membranes in the presence of Ca$^{2+}$ ions. The enzyme complex catalyzes efficient activation of factor X, and factor Xa in turn converts prothrombin to thrombin, which ultimately leads to successful formation of hemostatic plugs. In the classical cascade model of coagulation, factors VIII and IX are classified as components of the "intrinsic pathway" and are placed downstream of factor XI (Refs. 2 and 3; for recent review, see Refs. 4 and 5). However, it is now considered that the intrinsic pathway is insignificant in normal hemostasis, since individuals who lack initiators of this mechanism, i.e. factor XII (6), high molecular weight kininogen (7), and prekallikrein (8), are all asymptomatic. To today's best knowledge, the single mechanism that solely governs blood coagulation is the so-called "extrinsic pathway," which starts with the exposure of the extravascular component, tissue factor, to the bloodstream and formation of the active factor VIIa-tissue factor complex (VIIa-TF). The complex is able to activate factor X without a requirement for factors VIII and IX. Why, then, do hemophiliacs experience such severe hemorrhagic diatheses? This question was partly answered by the finding that VIIa-TF can activate not only factor X but also factor IX (9). Since then, evidence has accumulated for the indispensable roles of factors VIII and IX in the coagulation pathway initiated by tissue factor. Komiyama et al. (10) showed that factor IX is preferred to factor X as a substrate for VIIa-TF. More recently, Mann and co-workers (11, 12) demonstrated that the VIIa-TF-induced thrombin generation is strongly dependent on factor VIII. These studies clearly demonstrated the significance of factors VIII and IX. With these observations in mind, we attempted to validate the importance of these proteins considering a previously unrecognized factor, Mg$^{2+}$ ions.

Calcium ions (concentration in plasma: total, 2.2–2.6 mM; free, 1.1–1.3 mM) are the essential constituents of the coagulation cascade. However, participation of Mg$^{2+}$ ions (total, 0.8–1.2 mM; free, 0.4–0.6 mM) in this mechanism has been ignored.

Nonetheless, we found recently that folding of the native tertiary structure of factor IX requires not only Ca$^{2+}$ but also Mg$^{2+}$ ions (13). The Ca$^{2+}$-dependent binding of various conformation-specific probes was greatly enhanced by Mg$^{2+}$ ions at physiological concentrations even in the presence of excess Ca$^{2+}$ ions. This result suggests that factor IX has a specific binding site(s) for Mg$^{2+}$ ions that does not interact with Ca$^{2+}$ ions, and, moreover, that the binding of Mg$^{2+}$ ions promotes additional changes in the Ca$^{2+}$-bound conformation of the molecule. We failed to detect such Mg$^{2+}$-induced additional conformational changes in other vitamin K-dependent coagulation factors, such as factor X. Moreover, the Ca$^{2+}$-dependent activation of factor IX by the intrinsic pathway activator factor XIa was accelerated by Mg$^{2+}$ ions with a dramatic reduction in the apparent $K_m$ (13). Thus, the Mg$^{2+}$-induced additional conformational change appears to enhance the protein's ability to function. The activation by factor XIa is, however, not essential for physiological coagulation (11). Therefore, we examined the effect of Mg$^{2+}$ ions on the function of factor IX in the coagulation pathway initiated by VIIa-TF. Our results shed light on the indispensable roles of factors VIII and IX in vivo.

**EXPERIMENTAL PROCEDURES**

Materials—Plasma deficient in factor IX or factor VIII was from George King Biomedical. Phospholipids (phosphatidylcholine (PC) from egg yolk, phosphatidylethanolamine (PE) from egg yolk, and phosphatidylserine (PS) from bovine brain, all >98% pure), and bovine serum albumin (BSA) were from Sigma.

Proteins—The coagulation factors used were all of human origin. The following proteins were prepared by published methods: factor IX and factor X (14), factor Xa (15), factor Xa (16), and α-thrombin (17).
Factor XIa was a generous gift from Dr. Yutaka Komiyama of Kansai Medical University (Osaka, Japan). Factor VIIa and placentadervived tissue factor apoprotein were supplied by Dr. Tomohiro Nakagaki of Chemo-Sero Therapeutics Research Institute (Kumamoto, Japan). Recombinant factor VIII ("Kogenate") was a product of Bayer, Chemo-Sero Therapeutics Research Institute (Kumamoto, Japan). Recombinant factor VIIa and placenta-derived Factor XIa was a generous gift from Dr. Yutaka Komiyama of Kansai Medical University (Osaka, Japan).

Preparation of Phospholipid Vesicles and Reconstitution of Tissue Factor—Factor VIIa—Factor XIa Complexes—Phospholipid vesicles were mixed at desired stoichiometry and dried under N2 stream in a glass tube. They were suspended in 20 mM Tris-HCl, 140 mM NaCl, pH 7.5 (TBS), at 1 mg/ml, and were dispersed by sonication in a bath-type sonicator for 30 min. Incorporation of tissue factor apoprotein into the vesicles was achieved by a mixed detergent/phospholipid micelle technique (18). The stock solution of tissue factor in 2% Triton X-100 was diluted (to 0.01–1 nM) with TBS containing 5 mM Ca2+ and mixed with the suspension of phospholipid vesicles (typically 800 μM). The concentration of detergent was adjusted to 0.06%, and the mixture was incubated at 37 °C for 30 min. The suspension of reconstituted tissue factor was diluted appropriately with TBS plus 1 mg/ml BSA (TBS/BSA).

Clotting Assay—Pooled plasma was prepared by mixing citrated platelet-poor plasma from several normal healthy individuals. The plasma was dialyzed against TBS for 2 h at ambient temperature and stored frozen at −80 °C. Factor VIII- or IX-deficient plasma was dialyzed similarly prior to use. One part (50 μl) of the dialyzed plasma was mixed with one part of the phospholipid suspension and one part of the solution of coagulation initiator in TBS/BSA, and the mixture was incubated at 37 °C for 2 min. Clotting was initiated by the addition of one part of 10 mM Ca2+ (plus 4 mM Mg2+). The time required for clot formation was measured in an Amelung Coagulometer KC 4A. For VIIa-TF-induced clotting, plasma was preincubated with factor VIIa, and the reaction was initiated by the simultaneous addition of the suspension of reconstituted tissue factor and the Ca2+ (plus Mg2+) solution.

Chromogenic Assay for Factor Xa—The mixture of factor IXa, factor VIII, phospholipid vesicles (75% PC, 25% PS; w/w), and Ca2+ (plus Mg2+) in 80 μl of TBS/BSA was placed in a 96-well assay plate and equilibrated at 37 °C. Thrombin (10 μl) was added to 0.1 unit/ml to activate factor VIII, and incubation was continued for 1 min. Then the reaction was started by the addition of 10 μl of factor X and was terminated by the addition of EDTA after appropriate times (typically 5 min). Factor Xa was quantified by measuring its amidase activity with 4-bromoanilinophenyl-γ-Glu-Arg-p-nitroanilide (Seikagaku Kogyo, Tokyo, Japan) as substrate; the substrate was added to 0.5 mM, and the initial rate of p-nitroaniline liberation was monitored at 405 nm in a kinetic plate reader (Well Reader; Seikagaku Kogyo), with pure factor Xa as the standard.

Activation of Factor IX—Factor IX (0.1 μM in TBS/BSA) was incubated with 1 nM factor VIIa plus 0.1 nM tissue factor reconstituted in phospholipid vesicles (30 μM 75% PC, 25% PS) in the presence of Ca2+ (plus Mg2+) at 37 °C. The reaction was quenched at appropriate times by addition of 1/10 volume of 20 mM EDTA plus 1% Triton X-100. Detergent was used to terminate tissue factor activity that would otherwise affect the assay. Factor IXa was quantified by a specific clotting assay as described earlier (13).

RESULTS AND DISCUSSION

We examined the effect of Mg2+ ions on clotting. Pooled normal plasma was dialyzed to remove endogenous metal ions and the exogenously added anticoagulant, sodium citrate. It was then incubated with activated forms of coagulation factors and phospholipids. Clotting was initiated by addition of Ca2+ ions at concentrations close to physiological. When factor XIa was used as initiator, clotting times were reduced by Mg2+ ions. Approximately 10-fold lower concentrations of factor XIa were sufficient to yield the same clotting times as seen in the absence of Mg2+ ions (Fig. 1A). This result was reasonable because Mg2+ ions potentiate factor IXA activation by factor XIa (13). Next, we tested the effect of Mg2+ ions on factor IXA-induced clotting. Shorter clotting times were again obtained with Mg2+ ions (Fig. 1B). The apparent clotting activity of factor IXa was increased approximately 3-fold by Mg2+ ions. By contrast, the cation had a minimal effect on factor Xa-induced clotting (Fig. 1C). It was thus clear that Mg2+ ions accelerated not only factor IX activation by factor XIa but also factor X activation by factor IXa, while all the processes after the generation of factor Xa were unmodified. This result is consistent with our previous result that, among various vitamin K-dependent coagulation enzymes, only factor IX is responsive to Mg2+ ions (13).

We next investigated the activation of factor X by factor IXa in the presence of factor VIIa and phospholipids. Factor X at a physiological concentration (0.2 μM) was activated in the presence of various concentrations of Ca2+ ions (Fig. 2A). The activation rate at any Ca2+ concentrations was increased by Mg2+ ions, while Mg2+ ions alone were ineffective. When a 10-fold higher concentration of factor X was used, the amount of factor Xa generated and the effect of Mg2+ ions were essentially the same (data not shown). These results indicated that the physiological level of factor X was considerably higher than the Km value so that the reaction could proceed at maximum velocity (Vmax), and that Mg2+ ions increased the catalytic efficacy (kcat) of the enzyme. Indeed, the reported Km values for the IXa-VIIa complex (approximately 0.06 μM) are well below the plasma concentration of factor X (19, 20). When we varied the concentration of factor VIIa, a pronounced leftward shift of the required cofactor concentration was observed in the presence of Mg2+ ions (Fig. 2B). No such shift was observed upon varying the concentration of phospholipids (Fig. 2C). It appeared that Mg2+ ions augmented the coagulant activity of factor IXa by increasing the affinity of the enzyme for factor VIIa rather than for phospholipids. This result reflects the observed effect on catalytic efficacy, since it is known that factor VIIa increases the apparent Kcat while phospholipids decrease the apparent Km (19, 20).

We also evaluated the effect of Mg2+ ions on VIIa-TF-initiated coagulation, the main physiological pathway. We tested the effect on factor IX activation in a purified system. Factor IX at a physiological concentration (0.1 μM) was incubated with VIIa-TF and the factor IXa generated was quantified. The cation increased the rate of activation, and the velocity increased approximately 2-fold by Mg2+ ions under the experimental condition (Fig. 3). This effect of Mg2+ ions seemed rather small and, in plasma, the effect was more striking (see below). We are presently uncertain of the reason of this quantitative discrepancy but, nevertheless, it appeared that the cation also has a positive effect on factor IX activation.

We next investigated the effect of Mg2+ ions on VIIa-TF-induced clotting. Tissue factor, reconstituted on vesicles of 75% PC, 25% PS, and factor VIIa were added to dialyzed plasma. Clotting times were again decreased by Mg2+ ions in normal plasma and approximately 5 times lower concentrations of tissue factor was sufficient to yield the same clotting time (Fig. 4A). However, the cation was ineffective in factor IX- or VII-
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FIG. 2. Effects of Mg$^{2+}$ ions on the activation of factor X by factor IXa. A, dose-response to Ca$^{2+}$ ions. Factor X (0.2 μM) was incubated with 1 nM factor IXa in the presence of 2 units/ml thrombin-activated factor VIIa, 10 μM phospholipids (75% PC, 25% PS), and various concentrations of Ca$^{2+}$ (plus 1 mM Mg$^{2+}$) for 5 min. B, dose-response to factor VIIa. Factor X was activated as in A in the presence of the indicated concentrations of factor VIIIa. Concentrations of Ca$^{2+}$, Mg$^{2+}$, and phospholipids were fixed at 1 mM, 0.5 mM, and 30 μM, respectively. C, dose-response to phospholipids. Factor X was activated as in A in the presence of indicated concentrations of phospholipids. Concentrations of Ca$^{2+}$, Mg$^{2+}$, and factor VIIIa were fixed at 1 mM, 0.5 mM, and 10 units/ml, respectively. Detailed methods are given under “Experimental Procedures.” Closed circles, Ca$^{2+}$ alone; open circles, Ca$^{2+}$ + Mg$^{2+}$.

FIG. 3. Effects of Mg$^{2+}$ ions on the activation of factor IX by VIIaTF. Factor IX (0.1 μM) was incubated with 1 nM factor VIIa plus 0.1 nM tissue factor reconstituted on 30 μM phospholipid vesicles (75% PC, 25% PS) for indicated periods. The amount of factor IXa generated was quantified as described under “Experimental Procedures.” Closed circles, 1 mM Ca$^{2+}$ alone; open circles, 1 mM Ca$^{2+}$ + 0.5 mM Mg$^{2+}$.

deficient plasma (data not shown). These results suggest that the cation is indeed involved in the mechanism of physiologic coagulation through its action on factor IX. We also examined the effect of Mg$^{2+}$ ions with phospholipids of different composition. Gilbert and Arena (21) recently showed that PE, a constituent of natural procoagulant membranes (activated platelets), provides high affinity binding sites for factor VIII in membranes with low PS content and thereby facilitates efficient assembly of the IXa-VIIIa complex. We employed a preparation of phospholipids with a composition (75% PC, 20% PE, 5% PS) much closer to physiological than frequently used, and re-evaluated VIIaTF-induced clotting. This preparation was reported to be almost equipotent to the preparation of 75% PC, 25% PS with respect to the assembly of the active IXa-VIIIa complex (21). We expected that the contribution of factors VIII and IX would be emphasized and thus the effect of Mg$^{2+}$ ions would be more apparent.

Indeed, the effect of Mg$^{2+}$ ions was very striking with this preparation (Fig. 4B), and the cation again had minimal effects in plasma deficient in factor IX (Fig. 4C) or factor VIII (data not shown). Raising the concentration of tissue factor hardly decreased the clotting time without Mg$^{2+}$ ions (i.e. under conditions wherein the apparent coagulant activity of factor IX was attenuated), suggesting that the rate of generation of factor Xa should be reduced considerably. By contrast, factor Xa should be produced very rapidly in the presence of Mg$^{2+}$ ions. It appeared that VIIaTF-induced factor Xa generation is very strongly dependent on Mg$^{2+}$ ions and this action is solely mediated through the effects on factor IX/IXa. It is thus strongly suggested that most factor Xa is generated by IXa-VIIIa, not by the direct action of VIIaTF, in blood plasma where both Ca$^{2+}$ and Mg$^{2+}$ ions are present.

Magnesium ions potentiated all the factor IX-dependent processes in coagulation, and the cation appeared to be a crucial constituent of the coagulation cascade. Our data also underline the physiological significance of factors VIII and IX. The effect of Mg$^{2+}$ ions on the activation by factor IXa of factor X is especially noteworthy. The cation augmented the apparent catalytic efficacy of factor IXa by increasing the affinity of the protease for the cofactor. The plasma concentration of factor VIII is quite low (0.7 nM) as compared with those of factor IX (90 nM) and other components (11). Moreover, the active form, factor VIIIa, readily loses its activity either through spontaneous inactivation or through degradation by activated protein C (22, 23). Therefore, only minute amounts of factor VIIIa should be available during coagulation, and its availability seems to represent a critical bottleneck in the cascade. Our present observation that Mg$^{2+}$ ions facilitated efficient factor Xa generation with much lower amounts of factor VIIIa than those needed with Ca$^{2+}$ alone (Fig. 2B) effectively explains how the hemostatic process in vivo is guaranteed.

Although the cascade (or waterfall) theory (2, 3) constitutes a fundamental biochemical basis for the mechanism of coagulation and, in principle, it remains a viable theory after several revisions, erroneous descriptions in early cascade models still seriously influence the current models, found in many textbooks (e.g. Ref. 24). Because the original model was based on observations in test tubes rather than in vivo, it explains the mechanism of widely used diagnostic tests in vitro, i.e. APTT (activated partial thromboplastin time) and PT (prothrombin time). However, it does not reflect the actual hemostatic process. The most obvious discrepancy between the model and events in vivo is the role of factors VIII and IX, which have been
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![Figure 5: The main stream of the blood coagulation cascade: a revised hypothesis. This model focuses on the main pathway of the chain reaction of protease activation and stresses the action of Mg$^{2+}$ ions. Some important accessory reactions have been omitted for clarity. To reproduce the precise events that occur in vivo, we must consider the following: activation of factor X by VIIa-TF (see text), conversion of procofactors (V and VIII) to active cofactors, inactivation of factors Va and VIIIa via the thrombomodulin/protein C pathway (23), feedback activation of factor XI by thrombin (26, 27), neutralization of proteases by serpins, in particular by antithrombin III and tissue factor pathway inhibitor (27), and so on. We must also consider the effects of certain cellular mechanisms (in particular participation of platelets) and blood flow for an understanding of the dynamic hemostatic system. PL, phospholipids.]

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