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Influence of membrane composition on the enhancement of factor VIIa/tissue factor activity by magnesium ions

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Dear Sirs

A number of studies have shown that the γ -carboxyglutamate-rich (GLA) domains of vitamin K-dependent clotting proteins require Ca^{2+} to fold properly and bind to membranes (1, 2). Although plasma contains about 1.25 mM free Ca^{2+} and 0.5 mM Mg^{2+} (3), in vitro assays of clotting factor function often employ supraphysiologic Ca^{2+} concentrations (2.5 to 5 mM Ca^{2+}), and no Mg^{2+} . Sekiya and colleagues showed that Mg^{2+} enhances factor IX (fIX) structure and function in combination with physiologic Ca^{2+} concentrations (4, 5). Subsequent reports showed that, in the presence of plasma concentrations of Ca^{2+} , Mg^{2+} enhances the activity of factor VIIa (fVIIa) bound to tissue factor (TF) (6–10). The concept is that GLA domains typically bind seven or eight Ca^{2+} when it is the only divalent metal ion present (at supraphysiologic Ca^{2+} concentrations), but at plasma concentrations of Ca^{2+} and Mg^{2+} , two or three of these “calcium” binding sites are actually occupied by Mg^{2+} , with functional consequences (11). Mg^{2+} does not support clotting reactions in the absence of Ca^{2+} (12), also consistent with the notion that only a subset of the metal ion binding sites in GLA domains can be productively occupied by Mg^{2+} .

Many studies of TF activity have employed binary mixtures of phosphatidylserine (PS) and phosphatidylcholine (PC). We previously reported that phosphatidylethanolamine (PE) supports little procoagulant activity in PE/PC liposomes, but that PE dramatically reduces the PS requirement for TF:fVIIa activity (13, 14). More recently, we reported that factor VIIa binds preferentially to phosphatidic acid (PA) and that PA enhances rates of fX activation by fVIIa alone or in complex with the isolated tissue factor ectodomain (sTF) (15). However, these studies of phospholipid synergy were conducted at saturating Ca^{2+} concentrations without Mg^{2+} . Furthermore, the previous studies showing that Mg^{2+} enhances GLA domain function were typically performed with PS/PC liposomes or cell membranes. Therefore, the ability of Mg^{2+} to enhance fX activation by fVIIa has not been explored systematically as a function of phospholipid composition.

We now examine the ability of Mg^{2+} to enhance fVIIa function in the presence of PE or PA. Recombinant membrane-anchored TF (membTF) was reconstituted into liposomes of

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varying phospholipid composition (palmitoyl-oleoyl PC, palmitoyl-oleoyl PS, dioleoyl PE, and/or palmitoyl-oleoyl PA) and used to quantify rates of fX activation as previously described (15). We first measured fX activation by fVIIa on TF-liposomes composed of binary PS/PC mixtures. Consistent with studies cited above, physiologic concentrations of divalent metal ions (1.25 mM Ca^{2+} plus 0.5 mM Mg^{2+}) supported slightly higher rates of fX activation by membTF:fVIIa than did supraphysiologic Ca^{2+} concentrations (2.5 mM Ca^{2+} without Mg^{2+}) and substantially higher rates than with 1.25 mM Ca^{2+} without Mg^{2+} (Figure 1A, open diamonds, circles and squares, respectively), over the range of PS compositions tested. We replotted these data as normalized rates of fX activation in the presence of 1.25 mM Ca^{2+} plus 0.5 mM Mg^{2+} , relative to rates with either 1.25 or 2.5 mM Ca^{2+} (Figure 1B, open circles). The extent to which 0.5 mM Mg^{2+} enhanced fX activation decreased as the percent PS increased.

We next investigated the influence of 0.5 mM Mg^{2+} on PE/PS synergy in supporting fX activation by membTF:fVIIa. When membTF was incorporated into liposomes containing ternary mixtures of phospholipid (PS + PE = 30%; balance = 70% PC), PE strongly synergized with PS under all three conditions of divalent metal ions tested (closed symbols in Figure 1A; note left-shifted PS dependence). Thus, 0.5 mM Mg^{2+} enhanced rates of fX activation at all combinations of PS, PE and PC tested. Replotting the data as normalized rates (Figure 1B, closed squares) showed that 0.5 mM Mg^{2+} enhanced fX activation on membTF-liposomes containing PE, but to an extent that was somewhat blunted compared to membTF-liposomes without PE.

To determine if the effect of Mg^{2+} was simply additive to that of Ca^{2+} , we compared normalized rates of fX activation in two ways, using membTF-liposomes made with either binary PS/PC mixtures, or ternary PS/PE/PC mixtures. Rates of fX activation in mixtures of Ca^{2+} and Mg^{2+} , normalized to the same Ca^{2+} concentrations without Mg^{2+} (Figure 1C), showed that the effect of 0.5 mM Mg^{2+} was more pronounced at lower Ca^{2+} concentrations. Furthermore, liposomes with low PS contents were more affected by the addition of Mg^{2+} , compared to liposomes with high PS content. When fX activation rates in the presence of mixtures of Ca^{2+} and Mg^{2+} were normalized to Ca^{2+} concentrations that equaled the concentration of Ca^{2+} plus Mg^{2+} , the rate enhancements were less pronounced (Figure 1D). However, rates of fX activation in the presence of Ca^{2+} + Mg^{2+} were always higher than those with just Ca^{2+} .

PA enhances the proteolytic activity of fVIIa and sTF:fVIIa complexes (15). We prepared liposomes with binary PS/PC mixtures or ternary PS/PA/PC mixtures (PS + PA = 30%; balance = 70% PC), and tested the effect of mixtures of Ca^{2+} and Mg^{2+} on rates of fX activation by sTF:fVIIa (Figure 1E), using reaction conditions as previously described (15). When tested at 1.25 mM Ca^{2+} , we found that Mg^{2+} enhanced the rate of fX activation by sTF:fVIIa on membranes containing PA in a manner similar to the enhancement of fX activation by membTF:fVIIa in PS/PE/PC liposomes. Replotting these data as normalized rates (Figure 1F) showed that, similar to PE, the effects of Mg^{2+} are less pronounced when PA is incorporated in membranes along with PS.

This study shows that Mg^{2+} enhanced the rates of fX activation by fVIIa even when the phospholipid composition varied widely. The magnitude of the rate enhancement by Mg^{2+} was dependent on phospholipid composition, and was highest when PS was limiting. This is consistent with the idea that GLA domains bind more weakly, and less extensively, to membranes with low PS content (14, 16), and that Mg^{2+} enhances the affinity of GLA domains for such low-PS membranes. Interestingly, increasing the Ca^{2+} concentration up to 10 mM reduced the effect of Mg^{2+} but did not completely mask it. This was true even for relatively high PS contents or in the presence of PE, both of which otherwise tended to blunt the contribution of Mg^{2+} (Figure 1C and D). One should keep in mind that in these experiments, simultaneous binding of GLA domains of two clotting factors (fX and fVIIa) to phospholipid membranes are affected by mixtures of Ca^{2+} and Mg^{2+} . For membTF:fVIIa, rates of fX activation are dominated by the reversible membrane binding of fX, because fVIIa is captured efficiently by membTF under all phospholipid conditions tested (17). On the other hand, for sTF:fVIIa, the rates of fX activation are dependent on reversible membrane binding of both fX and fVIIa (as sTF:fVIIa).

Previous findings suggest that Mg^{2+} is better at stabilizing some of the metal ion binding sites in GLA domains than is Ca^{2+} alone (18), and the most solvent-accessible bound Ca^{2+} in GLA domains have been proposed to contribute directly to interactions with PS (14, 19). In mixtures of Ca^{2+} and Mg^{2+} , two of these solvent-accessible metal ion binding sites are known to be occupied with Mg^{2+} (11). Our results are therefore consistent with the idea that membrane binding of GLA domains is better stabilized when these “external” metal ion binding sites are occupied with Mg^{2+} rather than the Ca^{2+} .

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Figure 1. Magnesium enhances fX activation by membTF:fVIIa or sTF:fVIIa as a function of phospholipid composition

A) Activation of fX by fVIIa on membTF-liposomes containing binary mixtures of PS and PC (open symbols); or containing ternary mixtures of PS, PE and PC in which the total amount of PS + PE equals 30% (balance = 70% PC; closed symbols; note that as the %PS increases, the %PE decreases in this plot). Reactions contained 1.25mM Ca²⁺ (□, ■); 2.5 mM Ca²⁺ (○, ●); or 1.25 mM Ca²⁺ + 0.5 mM Mg²⁺ (◇, ◆). Initial rates of fX activation were divided by the membTF:fVIIa concentration. B) Normalized rates of fX activation by fVIIa on membTF-liposomes (data replotted from panel A) for binary PS/PC mixtures (○)

and ternary PS/PE/PC mixtures (■). Rates of fX activation at 1.25 mM Ca^{2+} + 0.5 mM Mg^{2+} were normalized to rates with either 1.25mM Ca^{2+} (solid lines) or 2.5 mM Ca^{2+} (dotted lines). *Inset* shows the data for normalization to 2.5 mM Ca^{2+} on an expanded y-axis. C) and D) Normalized rates of activation of fX by fVIIa on membTF-liposomes with increasing Ca^{2+} concentrations in the presence or absence of 0.5 mM Mg^{2+} , using membTF-liposomes containing 5% PS/25% PE/70%PC (●), 20%PS/10%PE/70%PC (◆), or 30% PS/70%PC (□). In panel C, rates in the presence of varying Ca^{2+} + constant 0.5 mM Mg^{2+} were normalized to rates obtained at the same Ca^{2+} concentration but without Mg^{2+} . In panel D, rates in the presence of varying Ca^{2+} + constant 0.5 mM Mg^{2+} were normalized to rates obtained at Ca^{2+} concentrations that equaled the total divalent cation concentration in the numerator (e.g., rates at 1.25 mM Ca^{2+} + 0.5 mM Mg^{2+} were normalized to those at 1.75 mM Ca^{2+} without Mg^{2+}). (Absolute fX activation rates in this panel were $620 \pm 36 \text{ min}^{-1}$ for 30:70 PS:PC liposomes at 10 mM Ca^{2+} + 0.5 mM Mg^{2+} , and $140 \pm 3.7 \text{ min}^{-1}$ for 5:25:70 PS:PE:PC liposomes at 1.25 mM Ca^{2+} .) E) Activation of fX by sTF:fVIIa on liposomes containing binary mixtures of PS and PC (open symbols) or ternary mixtures of PS, PA and PC in which PS + PA = 30% (balance = 70% PC; closed symbols). Reactions contained 1.25mM Ca^{2+} (□, ■), 2.5 mM Ca^{2+} (○, ●), or 1.25 mM Ca^{2+} + 0.5 mM Mg^{2+} (◇, ◆). Initial rates of fX activation were divided by the sTF:fVIIa concentration. F) Normalized rates of fX activation by sTF:fVIIa on liposomes (data replotted from panel E) for ternary PS/PA/PC mixtures (◆). Rates of fX activation at 1.25 mM Ca^{2+} + 0.5 mM Mg^{2+} were normalized to rates with either 1.25mM Ca^{2+} (solid lines) or 2.5 mM Ca^{2+} (dotted lines). (In panels E and F, initial rates of fX activation by sTF:fVIIa were quantified using 100 nM fX, 10–100 pM fVIIa, 20 nM sTF, and 50 μM phospholipids. Initial rates of fX activation by fVIIa on membTF-liposomes were quantified using 100 nM fX, 1–5 pM fVIIa, and 500 pM membTF in liposomes with approximately 25 μM phospholipids. Buffer was 20mM HEPES pH 7.4, 150 mM NaCl, 0.1% bovine serum albumin, and the indicated Ca^{2+} and Mg^{2+} concentrations.) Data in all panels are mean $\pm$ standard error; $n = 3$ to 4.