Anticoagulant Venom and Mammalian Secreted Phospholipases A2: Protein- versus Phospholipid-Dependent Mechanism of Action

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Key Words
Anticoagulant · Secreted phospholipase A2 · Snake venom · Mammalian · Factor Xa · Factor Va

Abstract
Some venom and mammalian-secreted phospholipases A2 (sPLA2) have been described to exert an anticoagulant effect. This review will discuss and compare phospholipid-dependent versus protein-dependent mechanisms of action of these sPLA2 on the coagulation cascade. The importance of venom proteins, and of the study of their pharmacological effects, to explore the physiological functions of homologous mammalian proteins is also pointed out.

Introduction
Snake venoms contain numerous components provoking various pharmacological effects [1–3]. This review will focus on venom and mammalian secreted phospholipases A2 (sPLA2) that exert an anticoagulant effect. sPLA2 most likely affect blood coagulation through hydrolysis of or binding to procoagulant phospholipids (PL) [4]. However, some data previously suggested that certain venom sPLA2 might exhibit a PL-independent mechanism [4]. The evidence of the existence of a protein-dependent mechanism of action came from studies on the basic sPLA2 isoform (CM-IV) of Naja nigriviridis venom [5], while the first demonstration of direct binding to a coagulation factor was obtained with the human group IIA sPLA2 (hGIIA). This sPLA2 binds to factor Xa leading to a deficiency in the formation of a fully active pro-
thrombinase complex [6]. Such a mechanism was also described to be involved for the CM-IV isoform [7]. The importance of a protein-dependent versus PL-dependent mechanism of action will be compared for different anticoagulant venom sPLA2. Anticoagulant effects of other recently cloned mammalian sPLA2 will also be discussed. Finally, these data highlight the interest of studying venom components in order to propose biological functions for homologous mammalian proteins.

Classification of Venom and Mammalian sPLA2

sPLA2 are enzymes found in mammals and animal venoms; they hydrolyse the fatty acids esterified at the sn-2 position of PL. They share common characteristics including low molecular mass (14–17 kD), calcium-dependent catalytic activity, the presence of 5–8 disulphide bridges and a number of conserved amino acids located in the calcium-binding loop and the catalytic site [8, 9]. Venom sPLA2 from Elapidae and Hydrophidae have been classified into group IA with the exception of a few enzymes that belong to group IB [10]. Venom sPLA2 from Crotalidae and Viperidae are found in group IIA and group IIB contains some sPLA2 from Bitis species. Finally, sPLA2 from bee and lizard venoms belong to group III. Despite common catalytic properties, venom sPLA2 differ greatly in their pharmacological effects such as neurotoxic, myotoxic, cardiotoxic or anticoagulant properties.

In mammals, different sPLA2 have been reported and classified into groups IB, IIA, IIC, IID, IIE, IIF, V, X and XII [9]. Their distribution is both tissue-specific, indicating different functions in different organs, and species-dependent, suggesting that a particular sPLA2 may not necessarily have the same function in all the species [9]. Although a function in the generation of eicosanoids has been proposed for some mammalian sPLA2, the precise biological function of each sPLA2 has still to be investigated.

Blood Coagulation Cascade

Platelet activation plays a central role in haemostasis and thrombosis, leading to the formation of the primary plug. In addition, the appearance of anionic PL at the membrane surface of activated platelets increases the efficiency of the coagulation process [11–13]. Since the plug formed by activated platelets is unstable, the initiation of the coagulation process is needed for the definitive blockade of bleeding. The coagulation process can be initiated by the extrinsic or intrinsic pathway, both leading to the activation of factor X to factor Xa (FXa) (fig. 1). Then, the prothrombinase complex, which is composed of FXa, factor Va (FVa), PL and calcium, constitutes an important step of the blood coagulation cascade leading to the generation of thrombin that converts fibrinogen into fibrin. The fibrin generated will therefore consolidate the primary plug. Three steps of the coagulation cascade require the formation of a complex between an active protease, a zymogen substrate and another protein cofactor on cell surfaces: the intrinsic tenase, the extrinsic tenase and the prothrombinase reactions. Because the assembly of these complexes occurs on a PL surface, these complexes may constitute potential targets for the action of anticoagulant sPLA2. However, the dependency on PL of the extrinsic tenase is more strict than for the prothrombinase and the intrinsic tenase since the two latter ones remain active when PL are replaced by lysoPL and fatty acids [14].
Anticoagulant Effect of Venom sPLA2

Some reviews discuss anticoagulant activities of venom sPLA2 and can be consulted for more details [2, 4]. Briefly, anticoagulant sPLA2 have been described in all major snake groups, i.e. Viperids, Crotalids, Elapids and Hydrophids. In Viperids, three sPLA2 from Vipera berus, Deboia russelli and Bitis caudalis have been described to be strongly anticoagulant. In Crotalids, Trimeresurus microsquamatus venom contains one strongly anticoagulant sPLA2. Venoms from Crotalus durissus terrificus, Agkistrodon halys blomhoffii and Agkistrodon halys pallas contain several sPLA2 isoforms, the most basic one being the most efficient in inhibiting blood coagulation. Other sPLA2 with anticoagulant activity have been isolated from the venoms of Bothrops asper, Bothrops atrox, Bothrops godmani and Bothrops jararacussu. In Elapids, the venom of N. nigricollis contains several sPLA2 isoforms, the basic one, CM-IV, accounting for the major anticoagulant effect. The basic sPLA2 from N. m. mossambica venom, designated as CM-III, is also a strong anticoagulant. Finally, the only sPLA2 from hydrophid venom described as weakly anticoagulant is found in Enhydrina schistosa.

While the anticoagulant activity of venom sPLA2 is well established, some controversies exist in the literature concerning the mechanism of their anticoagulant action. Some observations suggest that the sPLA2 catalytic activity is important, while others support the notion that the catalytic activity is not required. In fact, the mechanism of inhibition depends on each particular sPLA2 and therefore both hypotheses are correct.

PL-Dependent Mechanism of Anticoagulant Venom sPLA2

Since sPLA2 hydrolize PL, it has first been suggested that venom sPLA2 inhibited blood coagulation due to their ability to destroy pro-
coagulant PL. In such case, the higher effect should be on the extrinsic tenase since this complex is strictly dependent on the presence of integral procoagulant PL. An anticoagulant effect mediated by the hydrolysis of procoagulant PL has been reported in the case of the sPLA2 from *V. berus* [15], *A. h. pallas* [16], *B. jararacussu* [17], *N. n. atra* [18], and *T. mucrosquamatus* [19]. However, all sPLA2 have enzymatic activity and share the same residues involved in the catalytic reaction, but not all are anticoagulants. Therefore, it seems that the idea that an sPLA2 is an anticoagulant because it hydrolyses PL is too simplistic.

sPLA2 have different ability to penetrate the PL membrane, called the ‘penetrating ability’. This allows them to access and destroy the PL even in tightly packed membranes. It was proposed that the penetrating ability of sPLA2 was critical for exerting anticoagulant effects since this increases their efficiency to hydrolyse the procoagulant PL present on the surface of activated platelets or platelet-derived microvesicles [15].

Some sPLA2 have higher affinity for anionic PL (like phosphatidylserine), which mainly support the procoagulant activity. Therefore, the anticoagulant effect of these sPLA2 may be due to their competition with coagulation factors for binding to such PL and is not necessarily related to the hydrolysis [14, 20]. This scenario was proposed for the anticoagulant effects of sPLA2 from *T. mucrosquamatus* [19], *N. m. mossambica* [20] and *D. russelli* [21], *V. berus* [14] venoms. The difference between a PL hydrolysis and PL-binding mechanism is difficult to establish experimentally since both mechanisms may be used in combination by sPLA2. The use of chemically modified sPLA2 on the histidine48 of the active site in order to eliminate the catalytic activity is questionable, because the chemical group may modify the binding properties and/or the local conformation of the protein in addition to the suppression of its hydrolytic activity. The use of recombinant sPLA2 that will be modified by site-directed mutagenesis may be useful to better elucidate the PL-dependent mechanism.

It is interesting to note that all strong anticoagulant venom sPLA2 are usually basic proteins and the overall basicity of the sPLA2 seems to be necessary for their anticoagulant activity [15, 22]. However, not all basic sPLA2 are anticoagulants, supporting that a specific effect is involved in addition to the overall basicity. It was proposed that sPLA2 do have pharmacological sites in addition to the catalytic site to explain specific biological actions [23]. By sequence comparison analysis, the region 55–80, which is a basic exposed loop, appears important to explain the anticoagulant activity of venom sPLA2 and may constitute their ‘anticoagulant region’ [24]. This hypothesis has also been supported by the analysis of three-dimensional structures of few venom sPLA2 [22, 25], but no direct experimental evidence is available to support this hypothesis. The presence of this basic loop that seems to be related to strong anticoagulant action may either increase the penetrating ability or the binding affinity for anionic PL. The chemical modification of lysines (by carbamylation, acetylation or guanidination) only slightly affected the catalytic activity, but strongly decreased in the anticoagulant effect of sPLA2 from *D. russelli, B. asper, B. gadmani* and *N. nigricollis* venoms [21, 26, 27]. This indicates that the positive charges of lysines are important for the anticoagulant effect of sPLA2 and suggests that dissociation between hydrolytic activity and pharmacological properties of anticoagulant venom sPLA2 can be achieved.
Protein-Dependent Mechanism for the Anticoagulant Effect of the Basic CM-IV Isoform

The venom of *N. nigricollis* contains several sPLA2 isoforms, CM-I, CM-II and CM-IV. The CM-I and CM-II isoforms are weak anticoagulants and are likely to act mainly on the tenase complex due to PL hydrolysis [28, 29]. In contrast, the basic CM-IV isoform, which accounts for the major anticoagulant effect, inhibits both the tenase and prothrombinase reactions, and its anticoagulant effect was observed when only low PL hydrolysis was achieved [18]. Interestingly, when a chemically modified CM-IV was prepared in order to abolish the catalytic activity, it partially failed to inhibit the tenase reaction [29], but still remained a potent inhibitor for the prothrombinase complex [5]. Moreover, when the prothrombinase complex was reconstituted in the absence of PL, a strong inhibitory effect of CM-IV was observed. These observations offered the first evidence that the anticoagulant effect of CM-IV was due, at least partly, to a PL-independent mechanism on the prothrombinase. Recently, it was shown that CM-IV binds to bovine FXa but not prothrombin or FVa [7]. It forms a 1:1 complex with FXa with a $K_d$ of 500 nM. Moreover, increased amounts of added FVa to the mixture reverse the inhibitory effect on the prothrombinase complex, suggesting that CM-IV may compete with FVa for binding to FXa [7].

hGIIA has been detected in α-granules of platelets and is secreted within a few minutes upon activation by physiological agonists. Once secreted at the platelet plug, hGIIA is expected to adsorb to the plasma membrane rather than being diluted into the systemic circulation [30]. Moreover, it has been reported that other cell types (such as inflammatory cells) also secrete high amounts of hGIIA. Indeed, high concentrations of hGIIA have been detected at local sites, such as synovial and ascitic fluids and in tears, and localization of hGIIA appears to occur during local thrombolytic events [31].

hGIIA has been reported to exert an anticoagulant effect and might play a negative feedback role during the initiation of thrombosis upon secretion from activated platelets [32, 33]. hGIIA specifically inhibits the prothrombinase complex and this effect is still observed in the absence of phospholipids [6]. hGIIA forms a 1:1 complex with FXa ($K_d$ of 230 nM) and prevents the formation of the FXa/FVa complex [6]. Wild-type enzyme, as well as the catalytically inactive H48Q hGIIA obtained by site-directed mutagenesis, efficiently delays the formation of thrombin measured in human platelet-rich plasma. These observations indicate that the anticoagulant effect of hGIIA occurs under conditions close to the physiological situation and is independent of its catalytic activity. This supports an inhibitory mechanism based on the interaction of hGIIA with FXa even when natural phospholipids from plasma and platelet membrane are present [34].

hGIIA contains 13 lysine and 10 arginine residues scattered over its entire surface that form cationic clusters. Several of these basic clusters lie on the surface of the molecule that contacts the lipid membrane, the so-called ‘interfacial binding surface’ (IBS) [35]. Mutations of these basic residues lead to a significant reduction in the ability of hGIIA to inhibit prothrombinase activity and to bind to FXa. Further, increased salt concentrations completely abolished the binding of hGIIA to FXa, supporting an important role for elec-
Table 1. Effect of various mammalian and venom sPLA2 on prothrombinase activity

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<tr>
<th>pl</th>
<th>Inhibition of prothrombinase activity (IC50, nM)</th>
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<tr>
<td></td>
<td>absence of PL</td>
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<tr>
<td>hGIIA</td>
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<td>hGIA</td>
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<td>mGIIA</td>
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<td>hGV</td>
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<td>rGV</td>
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<td>hGX</td>
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<tr>
<td>Ba IV</td>
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<tr>
<td>CB</td>
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<td>Vbb</td>
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<tr>
<td>CM-III</td>
<td>9.9</td>
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<td>Apis mellifera</td>
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a Ba IV sPLA2 from B. asper has not been entirely sequenced but has been shown to be basic. CB is the basic subunit of crotoxin from C. d. terrificus, Vbb the sPLA2 from V. berus, CM-III the basic isoform from N. mossambica venom, and A. mellifera refers to the sPLA2 of the bee venom A. mellifera. The data are reproduced from reference 34 with some additions.

It was observed that the naturally catalytically inactive basic sPLA2, Ba IV from B. asper venom, inhibits prothrombinase activity in the absence of PL, suggesting it may act through direct binding to FXa [34]. A similar inhibition of prothrombinase was also observed with the basic subunit, CB, of the crotoxin (table 1). Recent observations in our laboratory show that CB binds to FXa like hGIIA and CM-IV [Grazyna Faure, pers. commun.]. It is thus likely that some other basic venom sPLA2 inhibit blood coagulation through direct binding to FXa. The overall basicity of the molecule is not sufficient to explain the inhibitory effect through binding to FXa since the basic CM-III sPLA2 from N. m. mossambica, the sPLA2 from B. caudalis and Notechis scutatus scutatus are devoid of inhibitory effect or weakly inhibitory in the absence of PL (table 1). Similarly, the bee venom sPLA2 inhibits prothrombinase activity through a PL-dependent mechanism (table 1).

The recently cloned mammalian sPLA2 may also exert anticoagulant effects. Indeed, mammalian rGIIA, mGIIA, hGV, rGV and mGIIID sPLA2, were found to inhibit prothrombinase activity in the absence of PL with efficiency similar to that of hGIIA (table 1). This suggests that they may also act through direct binding to FXa. These sPLA2 are all basic proteins as noticed above for anticoagulant venom sPLA2. Amino acid alignment of the hGIIA with other mammalian sPLA2 shows that several of the basic residues shown to be involved in the hGIIA/FXa complex are conserved between basic sPLA2 ac-
Fig. 2. Model showing the mechanism of inhibition of the prothrombinase complex by the hGIIA and the venom CM-IV isoform. These sPLA2 bind to FXa with a \( K_d \) value of 200–500 nM (a). They can replace FVa in the normal prothrombinase complex (bottom left), resulting in an inactive sPLA2/FXa complex (bottom right). Interaction of the substrate prothrombin (PT) with normal prothrombinase complex (b) or with sPLA2/FXa complex (c) is shown. sPLA2/FXa complex binds to prothrombin with the same affinity as FXa and sPLA2 do not interact with prothrombin. This explains the inability of these two sPLA2 to interfere in the prothrombinase activity of FXa alone. Reproduced with permission from Kerns et al. [7].

tive on prothrombinase via a PL-independent mechanism [34]. It is however expected that the precise interaction site of other mammalian and venom sPLA2 is not identical to that of hGIIA. The basic residues of hGIIA which are involved in the interaction with FXa, although all located on the IBS, are distributed throughout the primary sequence of the protein. It is thus possible that basic residues located at different positions on the primary sequence of other sPLA2 may be involved in similar basic cluster patterns. Studies based on the molecular modeling of these sPLA2 will be useful to test this hypothesis. On the other hand, neutral or acidic mammalian sPLA2 like hGIB and hGX were unable to inhibit prothrombinase activity in the absence of PL (table 1). This further supports the critical role of basic residues for the PL-independent antiprothrombinase activity of sPLA2. In agreement with this observation, these two sPLA2 do not share with hGIIA most of the basic residues that are important for binding to FXa. Furthermore, the hGX inhibits the prothrombinase complex in the presence of PL only, indicating that its inhibitory effect is likely to be related to binding or hydrolysis of procoagulant PL (table 1). This raises the question of the circulating levels of these sPLA2 in blood and their possible roles in thrombolytic events. Thus, the data on the circulating levels of GIID, GV, or GX sPLA2 and their presence in blood platelets will be of interest.
Perspectives

A microcalorimetry and molecular docking approaches are currently under investigation in order to better characterize the thermodynamic parameters of the hGIIA/FXa interaction and to define the part(s) of the FXa involved in formation of the complex. Similar approaches, as well as the production of a cocrystal with FXa, could be used with CB and CM-IV to define the nature of the residues involved in the interaction with FXa. The proposal antiprothrombinase mechanism for hGIIA and CM-IV is presented on figure 2. The elucidation of the parts of FXa involved in such an interaction may help to better define the interaction between FVas and FXa, since these sPLA2 compete with FVas for binding to FXa. These sPLA2 are small proteins compared to FVas and may offer an alternative approach. Finally, the study of venom sPLA2 has led to the investigation of anticoagulant effect of some mammalian sPLA2. However, whether such inhibitory effects may occur under physiological/pathological conditions remains to be determined.

References

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