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15

Platelet phospholipases A_2

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Introduction

The major physiological role of platelets is in the cessation of bleeding following damage to the vasculature. Ordinarily, platelets circulate within intact blood vessels in a quiescent state, but undergo extremely rapid and powerful activation upon exposure to the subendothelial matrix leading to formation of a platelet aggregate or vascular plug. This rapid response is achieved by the stimulatory action of a range of diverse agonists including extracellular matrix proteins, e.g. collagen, and products of the coagulation cascade, e.g. thrombin. Activation is reinforced through release of agonists from platelet granules, notably ADP, and liberation of thromboxane A_2 (TxA_2), the major product of the metabolism of arachidonic acid (AA) in the platelet. The positive feedback action of ADP and TxA_2 is of direct clinical relevance. The Antiplatelet Trialists' Collaboration[1] published a summary of 20 randomized trials in 1988, concluding that antiplatelet therapy significantly reduces (by ~ 25%) the risk of cardiovascular death, non-fatal myocardial infarction and nonfatal stroke in patients with unstable angina or a past history of heart attack, transient ischemic attack, or stroke[2,3]. A follow-up report by the same group, confirmed the efficacy of platelet inhibition in a broader spectrum of pathological conditions[4]. More recently, the CAPRIE study has shown that the ADP receptor antagonist, clopidogrel, and aspirin have a similar therapeutic benefit in individuals with a history of thrombotic disease[5]. Aspirin, however, remains the drug of choice in the majority of cases primarily for reasons of cost.

The availability of AA is the rate-limiting step in the formation of TxA_2 in platelets. As will be presented in detail below, it is now well established that AA is released from membrane phospholipids in activated platelets mainly by the enzyme phospholipase A_2 (PLA_2) activity. PLA_2 hydrolyses the *sn*-2 ester of glycerophospholipids to release a free fatty acid (AA and others) and a lysophospholipid. PLA_2 would also be a very good target for development of antithrombotics. This potential is unlikely to be exploited because of the proven efficacy of aspirin as an antiplatelet agent. Aspirin is an irreversible inhibitor of platelet cyclooxygenase-1 (COX-1), and, because platelets lack a nucleus, the enzyme cannot be replenished within the lifetime of the cell. As a consequence, only a low dosage of aspirin is required to achieve full blockade of TxA_2 formation in platelets, whereas other cells can generate new enzyme within a matter of hours. This therefore restricts the major effects of the COX-1 inhibitor to the platelet. It is for these reasons that aspirin is preferred to other non-steroidal antiinflammatory agents in the prophylaxis of thrombotic-based disorders. Treatment with aspirin increases the metabolism of AA via 12-lipoxygenase and 15-lipoxygenase enzymes in platelets, but this appears to have little physiological significance as the metabolites of these two enzymes do not have a major influence on platelet activation.

Recent years have seen rapid developments in our understanding of the regulation of PLA_2 in platelets, and of the pathways that govern the metabolism of the liberated AA. In this chapter, we will describe the major forms of PLA_2 in platelets and their regulation and give only brief details on the metabolism of AA. Comprehensive reviews on the action of TxA_2 and other eicosanoids on platelet function have been published[6,7].

Metabolism of AA in platelets

AA is an essential fatty acid that is stored in the *sn*-2 position of membrane phospholipids. It is the most prevalent fatty acid in platelets, and is found in particularly high levels in phosphatidylcholine, phosphatidylethanolamine

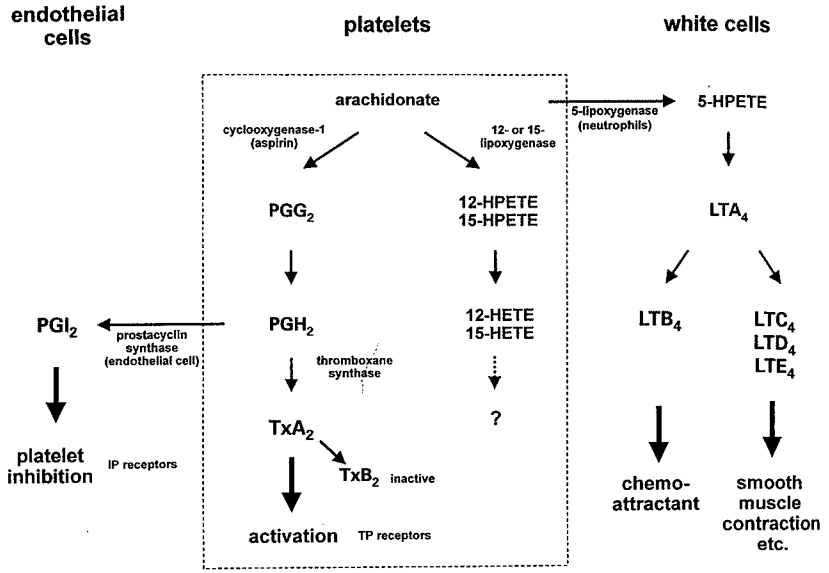

Fig. 15.1. Metabolism of AA in platelets and other cells. The metabolism of AA in platelets is shown in the dotted box. AA is converted by the action of cyclooxgenase-1 to the endoperoxides PGG_2 and PGH_2 and then to thromboxane A_2 (TxA_2), which stimulates platelet aggregation via the thromboxane receptor, TP, which is coupled to Gq-dependent activation of phospholipase C. PGH_2 is also released from the platelet and converted to the inhibitory prostaglandin, prostacyclin (PGI_2), in endothelial cells. PGI_2 inhibits platelet activation via the prostanoid receptor, IP, which is coupled to adenylyl cyclase. AA is also metabolized via 12- and 15-lipoxygenases to 12- or 15-hydroperoxyeicosatetraenoic acid (HPETE) and then to 12- or 15-hydroxyeicosatetraenoic acid. The physiological significance of this is uncertain as the products have no known functional roles in the platelet. The release of AA from platelets is available for conversion to proinflammatory leukotrienes by white cells.

and phosphatidylinositol. Phosphatidylcholine and phosphatidylethanolamine contain over 70% of the total AA in the cell. The AA-containing lipids are localized to the inner leaflet of the plasma membrane and membranes of intracellular organelles, with approximately similar levels in the two compartments. There is evidence that phosphatidylcholine and phosphatidylethanolamine, or possibly just the latter, are the major sources of AA following receptor stimulation (for discussion see[6,7]). Although AA is the predominant fatty acid in the *sn*-2 position of phospholipids, PLA_2s are able to hydrolyse phospholipids with other acyl chains. This is of particular relevance to dietary supplementation with fish oils enriched in ω-3 fatty acids, including eicosapentaenoic acid. The liberated eicosapentaenoic acid is converted to the inactive thromboxane A3, contributing to the antiplatelet effects of fish oil.

The AA that is liberated by the action of PLA_2 is available for metabolism via COX-1 or lipoxygenase pathways. COX-1 is the major AA metabolizing enzyme that is present in the platelet. In addition, platelets express 12-lipoxygenase and, in lower levels, 15-lipoxygenase, but lack the 5-lipoxygenase which is required for generation of leukotrienes (see Fig. 15.1). The major metabolite of AA in platelets is TxA_2. This is generated by the double oxygenation of AA yielding PGG_2 and then PGH_2, which, collectively, are known as the endoperoxides. PGH_2 is converted to TxA_2 by thromboxane synthase. TxA_2 is a powerful platelet agonist, inducing activation via the TP

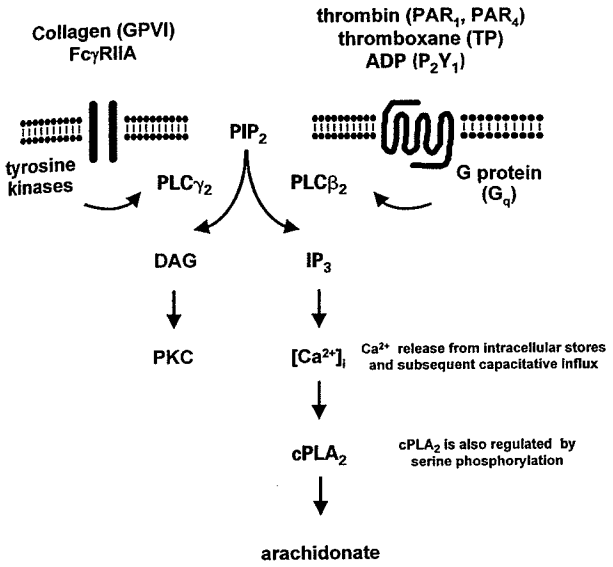

Fig. 15.2. Receptor liberation of AA in platelets. Many platelet agonists mediate activation through stimulation of phospholipase C (PLC) leading to formation of IP_3 and elevation of intracellular Ca^{2+}. These agonists can be subdivided into those which signal via tyrosine kinase-linked and G protein-coupled activation of PLCγ2 and PLCβ2, respectively. The newly generated Ca^{2+} activates $cPLA_2$-α. In addition, $cPLA_2$-α is regulated by phosphorylation on serines 505 and 727 via MAP kinases and MAP kinase-activated protein kinases (see text).

thromboxane receptor, which is coupled to activation of Gq and phospholipase C (Fig. 15.2). Because of the powerful activating effect of TxA_2, and beneficial effects resulting from conversion of released PGH_2 to prostacyclin in endothelial cells (see below), it was thought that inhibitors of thromboxane synthase would be good antiplatelet agents. These inhibitors would have the dual effect of prevention of TxA_2 generation and shunting of PGH_2 to prostacyclin in vascular endothelial cells. It has since been realized, however, that PGH_2 has reasonable activity at the TP receptor[8], making such inhibitors less effective than aspirin.

PGH_2 is also converted in much lower levels to PGD_2, PGE_2 and $PGF_2\alpha$. Platelets do not have specific receptors for PGE_2 and $PGF_2\alpha$, whereas the DP receptor for PGD_2 is present at a low level in some species. In general, the much lower levels of these three prostanoids means that they are of limited physiological significance in platelet function. The newly formed endoperoxides PGG_2/PGH_2 diffuse out of the platelet to the surrounding endothelial cell layer where they are converted to prostacyclin, one of the most powerful inhibitors of platelet activation. Prostacyclin activates IP prostanoid receptors on the platelet surface leading to elevation of cAMP. Physiologically, this helps to prevent progression of the platelet aggregate over the intact endothelium by acting as a physiological antagonist against most platelet agonists. Endothelial cells do not have thromboxane synthase and so cannot generate prostimulatory TxA_2.

The other product produced in reasonable levels in platelets following liberation of AA is 12-hydroxyperoxyeicosatetraenoic acid (12-HPETE) (Fig. 15.1). Although HPETE has no effect on its own, it has been reported to convert the response of subthreshold concentrations of

arachidonic acid to full aggregation at physiological concentrations[9]. This is mediated through potentiation of metabolism of arachidonic acid to thromboxane A_2. At higher, non physiological concentrations, HPETE inhibits platelet responses to arachidonic acid. In addition, HPETE is converted to 12-hydroxyeicosatetraenoic acid (12-HETE) which is released and metabolized to 12,20-HETE in neutrophils, where it may competitively inhibit metabolism of LTB_4[10]. Conversely, platelets convert neutrophil-derived leukotrienes into proinflammatory lipoxins[11]. There is also evidence that AA released from platelets is converted by 5-lipoxygenase to proinflammatory leukotrienes in white cells. The clinical significance of the interaction with neutrophils, however, is uncertain, but emphasizes the potential for cross-talk with other cells.

There is limited evidence to suggest that AA is a second messenger in its own right, although the physiological significance of this remains unclear. For example, AA has been shown to activate protein kinase C in the platelet cytosol independent of Ca^{2+} and phospholipids[12].

Mammalian PLA_2s

There is a diverse collection of PLA_2s in mammals, some of which are secreted whilst others remain intracellular. Secreted PLA_2s ($sPLA_2$) are Ca^{2+}-dependent, disulfide-rich, 14-18 kDa enzymes. The first mammalian $sPLA_2$ to be identified was the group IB enzyme, also known as pancreatic $sPLA_2$. This $sPLA_2$ is found at high levels in pancreatic juice, where it has a well-known function in the digestion of dietary phospholipids[13], but also at lower levels in lung, liver, spleen, kidney, and ovary where it has been proposed to play a role in cell proliferation, acute lung injury, cell migration, and endotoxic shock[14–16]. The second mammalian $sPLA_2$ to be identified was the group IIA enzyme which is expressed at high levels during inflammation[17], and is the principal bactericidal agent against Gram-positive bacteria in human tears[18].

In addition to the above roles, it is becoming clear that $sPLA_2$s are involved in a diverse set of physiological functions[14,19–21]. In the last few years, six mouse and five human $sPLA_2$s structurally related to groups IB and IIA $sPLA_2$s (mouse groups IIC, IID, IIE, IIF, V, and X, and human groups IID, IIE, IIF, V, and X) have been identified[22–27]. All of these group I/II/V/X $sPLA_2$s have similar primary structures, including identical catalytic site residues and partially overlapping sets of disulfides[28]. However, they are not closely related isoforms since the level of amino acid identity is typically 20-50% among these $sPLA_2$s. More recently, a novel human group III $sPLA_2$ was identified[29], which is structurally distinct from the group I/II/V/X $sPLA_2$s but related to the group III $sPLA_2$s found in bee and lizard venom. Very recently, a new human $sPLA_2$, group XII, was identified that is structurally distinct from all previously characterized mammalian and venom $sPLA_2$s and shares homology with known $sPLA_2$s only in short active site segments containing catalytic residues[30]. This diversity of $sPLA_2$ structures and the fact that the tissue distribution of the different $sPLA_2$s are distinct argue for a range of physiological functions for these lipolytic enzymes.

Mammalian cells also contain intracellular PLA_2s. Cytosolic PLA_2 ($cPLA_2$-α) is an 87-kDa protein that binds to membranes in a Ca^{2+}-dependent manner[31]. $cPLA_2$-α contains a C2 domain at its N-terminus which constitutes the Ca^{2+}-dependent membrane binding element[32–34]. Many of the $sPLA_2$s described above have been tested for *sn*-2 fatty acyl chain selectivity and are found to display little, if any, specificity for polyunsaturated versus saturated or monounsaturated chains[35]. In contrast, $cPLA_2$-α displays significant specificity for the *sn*-2 arachidonyl chain over the *sn*-2 oleate chain for example[35]. In response to a rise in intracellular calcium to the low micromolar range, $cPLA_2$-α transfers from the cytosol to the perinuclear region (nuclear membrane and what appears to be nearby membrane elements such as a portion of the Golgi and endoplasmic reticulum)[34,36,37]. The binding of $cPLA_2$-α to the intermediate filament protein vimentin may mediate this process[38]. Studies with $cPLA_2$-α-deficient mice provide strong evidence that $cPLA_2$-α plays a role in AA release for the biosynthesis of eicosanoids in inflammatory cells[39–42]. Two new human $cPLA_2$-α paralogs, $cPLA_2$-β and $cPLA_2$-γ, have been recently cloned based on searching genomic databases[43,44], and no physiological functions have been assigned to these enzymes.

Mammalian cells also contain an intracellular calcium-independent PLA_2 ($iPLA_2$)[45]. Studies using an $iPLA_2$ inhibitor as well as antisense technology suggest that this enzyme is involved in phospholipid remodelling in which the *sn*-2 chain of newly synthesized phospholipids is eventually replaced with a polyunsaturated fatty acyl chain[45]. $iPLA_2$ may not carry out this function in all mammalian cells[46]. $iPLA_2$ may be responsible for AA release seen in some mammalian cells undergoing apoptosis[47]. Multiple $iPLA_2$ splice variants have been identified[48] as well as a human paralog, $iPLA_2$-γ[49], but the functions of these enzymes are not known.

PLA_2s in platelets

At the time of this writing, platelets are known to contain two PLA_2s, group IIA $sPLA_2$ and $cPLA_2$-α. Kramer and coworkers discovered human group IIA $sPLA_2$ by purifying it from platelets[50]. Partial amino acid sequence obtained from the purified enzyme led to isolation of the human group IIA $sPLA_2$ genomic clone. They also showed that human group IIA $sPLA_2$ is secreted in active form following stimulation of platelets with thrombin. Whereas pancreatic-type (group IB) $sPLA_2$ is secreted as an inactive form with a propeptide and requires proteolytic activation, human group IIA does not contain a propeptide, only a signal peptide. Early studies also showed that rat and rabbit platelets contain group IIA $sPLA_2$[51–53]. Intravenous injection of ADP into rats leads to a rapid rise in plasma PLA_2 activity probably coming from activated platelets[54]. This enzyme is known to bind tightly to heparin, and after secretion from platelets may absorb onto heparan sulfate proteoglycan present on the surface of vasculature endothelial cells. Eventually, group IIA $sPLA_2$ in plasma is taken up by the liver and degraded.

$cPLA_2$-α was also purified from human platelets, leading to sufficient material for partial amino acid sequencing and cloning[55]. Bovine and rabbit platelets are also reported to contain $cPLA_2$-α[56,57]. As will be discussed in detail below, this enzyme is activated in agonist-stimulated platelets by phosphorylation and by a rise in intracellular Ca^{2+}.

Role of $cPLA_2$-α in platelet AA release

Two lines of evidence suggest that $cPLA_2$-α is responsible for much of the AA release in platelets following stimulation with the physiological agonists thrombin and collagen. The first comes from the use of a $cPLA_2$-α-specific inhibitor $AACOCF_3$[58,59]. This compound is an AA analogue in which the COOH group is replaced with a trifluoromethyl ketone group $COCF_3$. It is thought that $AACOCF_3$ forms a stable hemiketal adduct with the active site serine of $cPLA_2$-α (Ser-228) that resembles the tetrahedral intermediate that forms from the attack of this serine onto the carbonyl carbon of the substrate ester undergoing hydrolysis. $AACOCF_3$ does not inhibit $sPLA_2$s, although it does inhibit $iPLA_2$[60]. Two independent studies show that low micromolar concentrations of $AACOCF_3$ block virtually all of the AA released in human platelets stimulated with thrombin and calcium ionophore[61,62]. The control compounds $AACOCH_3$ and $AACH(OH)CF_3$, which do not inhibit $cPLA_2$-α in vitro, fail to block AA release in platelets. $AACOCF_3$ was further reported to block downstream oxygenation of AA in platelets[62], suggesting it may also act as a competitive inhibitor of oxygenating enzyme COX-1.

The second line of evidence that $cPLA_2$-α is involved in platelet AA release comes from studies of the regulation of this enzyme (described in detail below). Studies in human platelets show that $cPLA_2$-α becomes phosphorylated and activated in platelets stimulated with a variety of agonists[63,64]. This post-translational modification occurs with the same time course as AA release in platelets. $cPLA_2$-α is also phosphorylated and activated in a variety of other mammalian cells[31,65]. Although $cPLA_2$-α-deficient mice are available[39,40], studies of AA release in $cPLA_2$-α-deficient platelets have not been reported.

Regulation of $cPLA_2$-α in platelets

It has been proposed that AA liberation in platelets may be regulated by GTP-binding protein(s) and protein kinase C. Such studies were performed with platelet-membrane fractions or permeabilized platelets, and, platelet activation was performed with non physiological agonists such as PMA or fluoroaluminate[66–69]. By contrast, studies on the regulation of $cPLA_2$-α by phosphorylation and by Ca^{2+} have been reported in intact platelets activated by physiological agonists such as thrombin and collagen. Regulation of $cPLA_2$-α by phosphorylation and Ca^{2+} has also been studied in several other mammalian cells[31]. In this part, we will mainly focus our review on the regulation of $cPLA_2$-α that occurs during platelet stimulation by thrombin and collagen.

Regulation of platelet $cPLA_2$-α by Ca^{2+}

Because of the pivotal role of intracellular Ca^{2+} in the regulation of $cPLA_2$-α via its C2 domain, it follows that agonists that increase the levels of the cation also bring about the activation of $cPLA_2$-α. For the majority of agonists, the increase in Ca^{2+} is mediated through IP_3-dependent release of Ca^{2+} from intracellular stores and capacitative Ca^{2+} entry. The increase in IP_3 is brought about by G protein-coupled surface receptors, which activate phospholipase $C\beta_2$ ($PLC\beta_2$) isoforms, and tyrosine kinase-linked receptors, which activate $PLC\gamma_2$ (Fig. 15.2). Examples of G protein-coupled receptors that activate $PLC\beta_2$ downstream of Gq include the thrombin receptors, PAR_1 and PAR_4, the ADP receptor, P_2Y_1, and the thromboxane receptor, TP. Additionally, it has been proposed that the liberation of G protein $\beta\gamma$ subunits by Gi-coupled receptors such as the ADP receptor, P_2Y_1, P_2Y_2 and a_2-adrenoceptor, potentiates activation of $PLC\beta_2$ by Gq, although direct evidence for this is lacking. The major tyrosine kinase-linked receptors in human platelets are the collagen receptor GPVI and the platelet low affinity immune receptor, FcγRIIA. The depletion of intracellular Ca^{2+} stores by IP_3 leads to influx of Ca^{2+} by capacitative entry

and a correspondingly greater increase in AA release. There is also emerging evidence for additional pathways of Ca^{2+} entry that are independent of IP_3 such as direct regulation of cation channels by tyrosine kinases[70]. There have been no studies describing the site of translocation of cPLA$_2$-α in the platelet, although it is noteworthy that studies in other cells have reported translocation to intracellular membranes[34,36,37]. It is of particular interest to investigate whether cPLA$_2$-α translocates to dense tubular membranes, the site of location of COX-1 and thromboxane synthase in platelets[71].

There is limited evidence that cPLA$_2$-α is regulated by receptors in platelets which do not elevate intracellular Ca^{2+}. The cytokine thrombopoietin potentiates platelet activation by a wide range of agonists, but has no stimulatory action on its own. Phosphorylation of cPLA$_2$-α has been proposed as the mechanism underlying the priming action of thrombopoietin[72]. This is not the only mechanism of priming, however, as thrombopoietin potentiates activation in the presence of cyclooxygenase inhibitors[73]. The physiological significance of the priming effect of the cytokine is unclear. There is limited evidence that the major platelet integrin, αIIb-β3, which is a receptor for fibrinogen and von Willebrand factor, can activate cPLA$_2$-α.

Regulation of cPLA$_2$-α by phosphorylation

Lin and coworkers at Genetic Institute in Boston were the first to provide strong evidence that cPLA$_2$-α is phosphorylated in mammalian cells in response to agonists that mobilize AA from phospholipids[74]. After this group cloned cPLA$_2$-α, they realized that the protein contains a consensus motif surrounding Ser-505 for phosphorylation by mitogen activated protein kinase family members (MAPKs). This group also provided strong evidence, but not proof, that cPLA$_2$-α is phosphorylated on Ser-505 when expressed in CHO cells. When the S505A cPLA$_2$-α mutant was expressed in CHO cells, AA release in response to Ca^{2+} ionophore and the protein kinase C activator PMA was greatly decreased compared to that produced by expression of wild-type cPLA$_2$-α. They also showed that cPLA$_2$-α phosphorylation led to a slight decrease in the electrophoretic mobility of the enzyme. This gel shift assay, which can be examined by immunoblotting methods using total protein extracted from mammalian cells, has been used by many investigators to track cPLA$_2$-α phosphorylation in response to agonists (> 300 published studies).

However, we were never completely satisfied with the gel shift assay because it does not provide site-selective cPLA$_2$-α phosphorylation data. We were able to isolate sufficient quantities of cPLA$_2$-α from a baculovirus/insect cell expression system to determine the sites of phosphorylation by protein chemical methods. We determined that cPLA$_2$-α was phosphorylated not only on Ser-505 but also on serines-437, 454, and 727[75]. This was accomplished by using combined HPLC/electrospray mass spectrometry to fully sequence the four tryptic phosphopeptides. Radiometric methods with ^{32}P were used to show that these four phosphorylations accounted for all of the cPLA$_2$-α phosphorylation in these cells. We then studied cPLA$_2$-α phosphorylation in human platelets following stimulation with the physiological agonists thrombin and collagen. Although sufficient amounts of cPLA$_2$-α from mammalian cells for mass spectrometry studies cannot be readily obtained, we were able to use ^{32}P-radiometric methods to show that the radiolabelled tryptic peptides obtained from immunoprecipitated platelet cPLA$_2$-α comigrated with authentic phosphopeptides (prepared by solid-phase peptide synthesis) on both a reverse-phase HPLC column and on a two-dimensional cellulose plate (electrophoresis followed by thin layer chromatography). By this method, we showed that cPLA$_2$-α is phosphorylated in thrombin and collagen stimulated platelets on Ser-505 and on Ser-727, and this accounted for all of the phosphorylation in these cells (Ser-437 and Ser-454 phosphorylations were not observed)[76] (Fig. 15.3). Since cPLA$_2$-α phosphorylation is stoichiometric in platelets (full gel-shift) and since the cpm incorporated at Ser-505 and Ser-727 are virtually identical, we conclude that platelet cPLA$_2$-α becomes stoichiometrically phosphorylated on both serines in response to thrombin. We also showed that cPLA$_2$-α is phosphorylated exclusively on Ser-505 and on Ser-727 in HeLa cells stimulated with activators of the stress-activated protein kinases (SAPKs, members of the MAPK family)[76]. These are the only published studies reporting the rigorous mapping of cPLA$_2$-α phosphorylation sites. Our work cast doubt on the suggestion, based only on immunoblot analysis, that cPLA$_2$-α is phosphorylated on tyrosine in HeLa cells[77].

We, along with Kramer and coworkers, showed that the p38 member of the SAPK family or a closely related kinase and not the p42/p44 MAPKs (ERK1 and 2) was responsible for cPLA$_2$-α phosphorylation in platelets[78–81]. We found that the inhibitors of p38, SB202190 and SB203580, blocked about 50% of the phosphorylation on both Ser-505 and Ser-727 in thrombin stimulated platelets. Since the Ser-727 site does not contain the critical residues for recognition by MAPKs, the inhibition data suggests that the kinase responsible for Ser-727 phosphorylation is activated by p38. The fact that cPLA$_2$-α phosphorylation is only 50% inhibited by SAPK inhibitors suggests that more than one isoform of p38 (inhibitor sensitive and insensitive

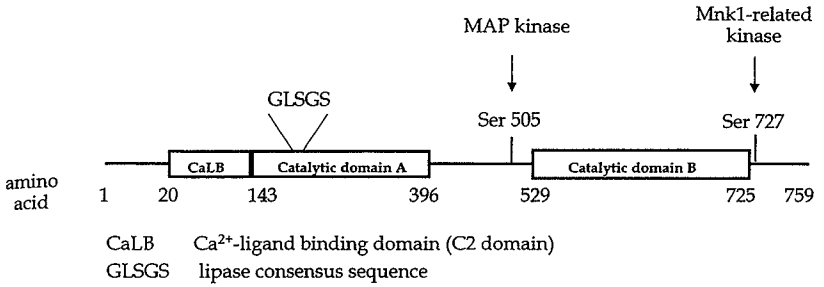

Fig. 15.3. Schematic representation of major structural domains of the cPLA$_2$-α. The catalytic domain composed of two parts of the protein (catalytic domain A and catalytic domain B), the Ca^{2+}-binding domain are indicated as well as two phosphorylation sites.

forms) may be phosphorylating cPLA$_2$-α in platelets. Since these studies, three new p38 isoforms have been cloned[82–85], bringing the current count to four gene products (SAPK2a/2b/3/4). Using recombinant SAPKs and specific antibodies, we went on to show that all four SAPKs phosphorylate cPLA$_2$-α in vitro at Ser-505[78]. All four SAPKs are present in platelets and SAPK2a/2b and four undergo activation in thrombin-stimulated platelets[78]. However, SB202190 and SB203580 inhibit SAPK2a/2b but not SAPK3/4[86]. Thus, it seems that the partial inhibition of cPLA$_2$-α phosphorylation by the SB202190/SB203580 inhibitors may be due to inhibitor sensitive and insensitive forms of SAPKs that phosphorylate cPLA$_2$-α upon thrombin addition. Alternatively, the SB202190/SB203580-insensitive phosphorylation could be mediated by other members of the MAPK family such as ERK5 or Jnk. In collagen stimulated platelets, only the SB202190/SB203580-sensitive SAPKs, SAPK2a/$_2$b, become activated, and we found that these inhibitors block all of the cPLA$_2$-α phosphorylation (Ser-505 and Ser-727) induced by collagen[78]. We then went on to show that complete blockage of cPLA$_2$-α phosphorylation leads to a shift in the AA release vs. collagen concentration dose–response curve in platelets. Threefold higher amounts of collagen are needed to elicit the same AA release response in the absence of cPLA$_2$-α phosphorylation (i.e. with SB202190/SB203580 present) as in the presence of phosphorylation[78]. We also showed that stress stimuli that activate platelet SAPKs (oxidants and osmotic shock) lead to enhanced cPLA$_2$-α phosphorylation and AA release in platelets[87], consistent with a role of SAPK-catalyzed cPLA$_2$-α phosphorylation in augmenting AA release in platelets.

Kramer et al. showed that phorbol ester activation of the p42/p44 MAPK members in platelets leads to cPLA$_2$-α phosphorylation, and we showed that such phosphorylation also occurs on Ser-505 and Ser-727[65]. It should be noted that this cPLA$_2$-α phosphorylation pathway is an induced response to the non-physiological phorbol ester agonist; as noted above, cPLA$_2$-α phosphorylation is catalysed by SAPKs in response to the physiological agonists thrombin and collagen.

Our studies with SAPK inhibitors SB202190/SB203580 led us to propose that the kinase responsible for phosphorylation of cPLA$_2$-α on Ser-727 is activated by one or more SAPKs. The identification of MAPK-activated kinases is rapidly advancing, and we decided to test all of the kinases known at the time of our study to be activated by both p42/p44 MAPKs and by SAPKs (MNK1[88,89], PRAK1[90], MSK1[91], MAPKAP-K2[89], and MAPKAP-K3[92]) for their ability to phosphorylate cPLA$_2$-α in vitro. MAPKAP-K1 (p90^{rsk}) is not activated by SAPK2a, but lies downstream of ERK2[86,93] and thus was not considered further. Using mass spectrometry, we proved that MNK1 phosphorylates cPLA$_2$-α in vitro uniquely at Ser-727 and that PRAK1 and MSK1 produce a radiophosphorylated tryptic peptide that co-migrates with authentic Ser-727 phosphorylated tryptic peptide on HPLC and on a two-dimensional cellulose plate[65]. MAPKAP-K2 and MAPKAP-K3, although enzymatically active, did not phosphorylate cPLA$_2$-α in vitro. Using specific antibodies, we showed that MNK1 and PRAK1, but not MSK1, are present in platelets and become activated upon thrombin addition and with the same time course as activation of SAPKs and cPLA$_2$-α phosphorylation[65]. At this point it seems clear that cPLA$_2$-α phosphorylation on Ser-727 in activated platelets is carried out by MNK1, PRAK1, or a closely related isoform.

Having defined that pattern of cPLA$_2$-α phosphorylation in platelets and the family of kinases responsible for the modifications, we further examined the functional consequence of Ser-505 and Ser-727 cPLA$_2$-α phosphorylation[65]. We constructed a mammalian cell expression plasmid containing mouse cPLA$_2$-α cDNA and prepared several clones of stably transfected CHO cells, HeLa cells, and HEK293 cells (human embryonic kidney endothelial cells). Clones were also established that expressed the phosphorylation site mutants (S505A, S727A, and S505A/S727A). We studied cPLA$_2$-α phosphorylation in these cells by radiolabelling with ^{32}P-phosphate and examining the radioactive tryptic peptides derived from immunoprecipitated cPLA$_2$-α by HPLC and 2-dimensional cellulose chromatography. We found that cPLA$_2$-α was phosphorylated stoichiometrically only on Ser-505 and Ser-727 in all three cell types and that mutation of these serines to alanines abolished phosphorylation, thus confirming our phosphorylation site mapping studies.

We next quantified AA release in these clones in response to agonist stimulation[65]. Several clones expressing each type of cPLA$_2$-α were selected for study based on comparable amounts of expressed cPLA$_2$-α (based on immuno- and RNA-blots)[65]. Similar results were obtained with CHO, HeLa, and HEK293 cells[65]. Expression of wild-type cPLA$_2$-α in CHO cells leads to an increase in AA release compared to the non-transfected parental cells. Stimulation with an intermediate concentration of Ca^{2+} ionophore alone leads to more AA release than stimulation with the protein kinase C agonist PMA alone, and there is a synergistic effect when both agonists are added. These results are similar to those reported by Lin et al. for cPLA$_2$-transfected CHO cells[74]. Mutation of either Ser-505 or Ser-727 of cPLA$_2$-α significantly reduces AA release, and the double mutant fails to produce AA above the level made by non-transfected cells. When a high concentration of Ca^{2+} ionophore is used, the effect of double mutation on AA release is rescued showing that the mutant can be fully functional in these cells. These results establish that phosphorylation of cPLA$_2$-α on Ser-505 and on Ser-727 are important for efficient AA release and that high Ca^{2+} concentrations can overcome the need for cPLA$_2$-α phosphorylation. Finally, we prepared HEK293 cell double transfectants that overexpress wild-type cPLA$_2$-α and a dominant negative form of MNK1[94]. Expression of dominant negative MNK1 led to a significant reduction in AA release, but when HEK293 cells were stimulated with high Ca^{2+} ionophore concentration, no effect of dominant negative MNK1 was observed[65]. These results support our earlier studies that cPLA$_2$-α is phosphorylated by MNK1 or a closely related kinase in platelets and that phosphorylation of cPLA$_2$-α is not required for AA release in the presence of high intracellular Ca^{2+}. Our studies also underscore the need for rigorous analysis of cPLA$_2$-α phosphorylation using site-selective protein chemical techniques rather than simply relying on gel shift analysis.

In the early studies by Kramer et al. it was shown that fully phosphorylated cPLA$_2$-α partially purified from thrombin-stimulated human platelets is about two- to threefold more active than non-phosphorylated enzyme toward the hydrolysis of phospholipid vesicles in vitro[95]. Phosphorylation is unable to bring about activation of cPLA$_2$-α on its own, but increases the response to elevation of Ca^{2+}. This can be explained by the ability of Ca^{2+} to stimulate movement of cPLA$_2$-α to intracellular membranes, whereas phosphorylation increases the catalytic efficiency of the membrane-bound enzyme. In support of this, mutational studies have shown that phosphorylation of serine-505 does not play a role in translocation to intracellular membranes[37]. Thus, in combination, Ca^{2+} and phosphorylation induces a larger increase in activity than either stimulus on its own.

Evidence that group IIA sPLA$_2$ may not be involved in AA release in platelets stimulated with physiological agonists

Group IIA sPLA$_2$ has been detected in α-granules of platelets from different species including rat, rabbit and human and is secreted within a few minutes upon activation by physiological agonists[50,96,97]. The question of the possible involvement of group IIA sPLA$_2$ in AA liberation during platelet activation has been investigated by two different approaches. First, addition of a specific inhibitor for group IIA sPLA$_2$ did not inhibit AA liberation from thrombin-stimulated platelets, while specific inhibition of cPLA$_2$-α led to a strong decrease in such a liberation[61]. Secondly, it was shown that group IIA sPLA$_2$-depleted platelets aggregated as much as control platelets and produced similar amounts of TxA$_2$ upon agonist stimulation[97]. These data lead to the conclusion that group IIA sPLA$_2$ does not play a significant role in AA liberation upon agonist stimulation. Natural strains of mice lack group IIA sPLA$_2$[98], and it would be interesting to examine AA and TxA$_2$ release in platelets derived from these strains.

Since thrombosis and inflammation are two closely related processes, it is possible that once released from platelets, group IIA sPLA$_2$ could participate in eicosanoid and/or biologically active lysophospholipid formation in other inflammatory cells or from microvesicles and therefore lead to the propagation of the inflammatory process[99,100]. The addition of group IIA sPLA$_2$ on A23187-treated granulocytes, IgE/antigen-primed mast cells, TNFα-stimulated HUVEC and BRL-3A cells, leads to more prostanoids generated than that elicited by each agonist alone[101–104]. Moreover, group IIA sPLA$_2$ does contribute to AA release for eicosanoid formation in epithelial and fibroblastic cells[20]. In these cells, group IIA sPLA$_2$ seems to contribute to AA release during the late phase of eicosanoid formation, typically tens of minutes to hours after cell activation, whereas cPLA$_2$-α contributes to the early phase of AA release. In this context, it is important to note that platelets release AA and TxA$_2$ within a few minutes after stimulation by physiological agonists. Once secreted from platelets, group IIA sPLA$_2$ is expected to adsorb to the plasma membrane of nearby cells, rather than being diluted into the systemic circulation. This is due to its ability to bind heparan sulfate proteoglycan present at the cellular surface[20,105,106]. In fibroblastic cells, group IIA sPLA$_2$ anchored to cell surface heparan sulfate proteoglycan is internalized in a process that leads to AA release coupled to eicosanoid formation[106]. In contrast, with mast cells, such anchoring leads to group IIA sPLA$_2$ internalization for degradation[107].

The ability of exogenous added group IIA sPLA$_2$ to release AA from platelets was also studied. Addition of micromolar concentrations of exogenous enzyme to resting platelets does not induce platelet aggregation, AA liberation, or plasma membrane phospholipid hydrolysis[61,108,109]. It therefore appears that the extracellular face of the platelet plasma membrane is a poor substrate for group IIA sPLA$_2$. This is probably due to the fact that this membrane face is highly enriched in sphingomyelin and phosphatidylcholine[110,111], and that group IIA sPLA$_2$ binds extremely weakly to vesicles of zwitterionic phospholipids[112]. Phosphatidylserine is known to be transferred to the extracellular face of the plasma membranes of platelets during cell activation. However, this change in membrane composition is not sufficient to allow group IIA sPLA$_2$ to act since little or no AA is released when activated platelets are treated with exogenous addition of group IIA PLA$_2$[61,109]. Addition of phosphatidylserine to phosphatidylcholine vesicles leads to enhanced binding of group IIA sPLA$_2$ in vitro, but interfacial binding of enzyme remains weak unless the concentration of acidic phospholipid lipid approaches 100 mole per cent (M.H. Gelb, unpublished observations).

One possible hydrolytic action of the group IIA sPLA$_2$ is on microvesicles shed from platelets during activation by thrombin and collagen[108,113]. Such microvesicles are enriched in phosphatidylserine and phosphatidylethanolamine in the outer leaflet. Micromolar concentrations of group IIA sPLA$_2$ induced a modest phospholipid hydrolysis on such microvesicles after one-hour incubation, while a marked hydrolysis was observed in the presence of sphingomyelinase[108]. The physiological relevance of such a process remains to be established, but it is unlikely it would contribute significantly to AA liberated from platelets during thrombin or collagen stimulation. The shedding of microvesicles is a slow process, whereas AA release is complete within a few minutes[114,115]. In addition, weak agonists which are unable to induce microvesicle shedding are potent inducers of eicosanoid formation during platelet activation[115,116]. However, the possibility that physiologically relevant signaling lipids including lysophosphatidic acid and lysophosphatidylcholine are produced during group IIA sPLA$_2$-catalysed microvesicle hydrolysis needs to be considered.

In conclusion, available evidence goes against a role for group IIA sPLA$_2$ in generating AA during physiological agonist stimulation of platelets. In light of the fact that several new sPLA$_2$s have been discovered in recent years (discussed above), it will be important to explore the presence of these enzymes in platelets. It should also be mentioned that the action of an sPLA$_2$ other than the group IIA enzyme could contribute to AA release during platelet activation. It has been shown that the action of cPLA$_2$-α is needed as a prerequisite for sPLA$_2$ action in mast cells[117] and in fibroblasts[118]. This complicates the interpretation of the results that AACOCF$_3$ blocks virtually all of the AA released in activated platelets, but the results with this inhibitor show that cPLA$_2$-α plays the dominant role in liberating AA from platelet membrane phospholipids for the biosynthesis of eicosanoids.

Possible physiological functions of platelet-derived group IIA sPLA$_2$

While the synthesis and the subsequent secretion of group IIA sPLA$_2$ via the classical secretory pathway in many cell-types is regulated by proinflammatory stimuli[20,119,120] (Il-1, Il-6, TNF, LPS), in some cells group IIA sPLA$_2$ is rapidly released from storage granules following cell activation. For example, group IIA sPLA$_2$ is stored in α-granules of resting platelets and released along with other granule proteins during activation. Such a secretion is correlated with the activation state of blood platelets and with the efficiency of the release reaction, but specific regulation of group IIA sPLA$_2$ secretion compared to other granule proteins has not been reported. It is likely that platelet-derived group IIA sPLA$_2$ mainly exerts its physiological functions once secreted since no report of an action of group IIA

Table 15.1. The possible biological functions for platelet-derived group IIA $sPLA_2$

Reported properties for group IIA $sPLA_2$	Expected biological functions for platelet-derived group IIA $sPLA_2$
Binding to heparan sulfate proteoglycans	Extracellular or intracellular participation in AA production by cells other than platelets
Intracellular degradation of bacteria, extracellular hydrolysis of bacterial phospholipids	Antimicrobial effect
Binding to blood coagulation factor Xa	Anticoagulant effect
Platelet-derived microvesicle hydrolysis	Production of biologically active lipids (LPA, LPC), removal of microvesicles
LDL hydrolysis	Proatherogenic effect
Membrane hydrolysis of cells with a loss in the membrane asymmetry	Removal of apoptotic/injured/activated cells

$sPLA_2$ inside platelets has been described. Group IIA $sPLA_2$ binds tightly to heparin[20,106]. It is possible that it forms a complex with heparin inside of granules which prevents it from acting on granular membranes, but this idea remains to be investigated. It is still a question whether platelet-derived group IIA $sPLA_2$ may account for the high increase in the group IIA $sPLA_2$ level found in serum during inflammatory diseases. In the rat, in vivo platelet activation by administration of ADP was followed by a transient rise in plasma $sPLA_2$ activity[54]. However, it has been reported that other cell-types (such as inflammatory cells and liver cells) also secrete high amount of group IIA $sPLA_2$[20,121]. Group IIA $sPLA_2$ is expected to stay, at least partly, at the cellular surface after secretion (due to its proteoglycan binding properties) rather than being diluted into the systemic circulation[105,106]. High concentrations of group IIA $sPLA_2$ have been detected in local sites, such as synovial[122] and ascitic fluids[123] and in tears[18], and localization of group IIA $sPLA_2$ appears to occur during local thrombolytic events[20,108]. The possible physiological functions of the group IIA $sPLA_2$ will be reviewed mainly with the working hypothesis that it is acting locally and extracellularly after secretion from platelets (Table 15.1). However, an intracellular action of the group IIA $sPLA_2$ after binding to the cell surface and subsequent internalization has been proposed in some cells and discussed above regarding the participation of platelet-derived group IIA $sPLA_2$ in eicosanoid formation by other cells[106]. Finally, it has to be considered that group IIA $sPLA_2$ may also be secreted by other cells present at the thrombus foci and therefore act with the platelet-derived enzyme.

Anticoagulant effect of group IIA $sPLA_2$

The group IIA $sPLA_2$ has been reported to exert an anticoagulant effect and may play a negative feedback role during the initiation of thrombosis once secreted from activated platelets[124,125]. Group IIA $sPLA_2$ specifically inhibits the prothrombinase complex composed of factor Xa (FXa), factor Va (FVa), phospholipids and calcium, and this inhibitory effect is still observed in the absence of phospholipids[125,126]. Group IIA $sPLA_2$ forms a 1:1 complex with FXa and prevents the formation of the FXa/FVa complex[125]. Group IIA $sPLA_2$ 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'[127]. Mutations of these basic residues led to a significant reduction of the ability of group IIA $sPLA_2$ to inhibit prothrombinase activity and to bind to FXa, and, increased salt concentrations completely abolished the binding of group IIA $sPLA_2$ to FXa, supporting an important role of electrostatic interactions[128]. High concentrations of FVa reversed the inhibitory effect of group IIA $sPLA_2$ on the prothrombinase complex, suggesting that group IIA $sPLA_2$ may compete with FVa for binding to FXa. Therefore, group IIA $sPLA_2$ is expected to act at early stages of the coagulation process by delaying the formation of a fully active prothrombinase complex, until enough FVa is generated.

Wild-type enzyme, as well as the catalytically inactive H48Q group IIA $sPLA_2$, efficiently delays the formation of thrombin measured in human platelet-rich plasma (PRP). Therefore, the anticoagulant effect of group IIA $sPLA_2$ occurs under experimental conditions close to the physiological situation and is independent of its catalytic activity, even when natural phospholipids from plasma and platelet membrane are present, supporting an inhibitory mechanism based on the interaction of group IIA $sPLA_2$ with FXa[128]. As discussed above, the levels of group IIA $sPLA_2$ in serum strongly increase from 0.35 nM up to 600 nM under various inflammatory states[121,129], and, high concentrations are expected to be found at local inflammatory/thrombolytic sites. Group IIA $sPLA_2$ inhibits the prothrombinase activity with an IC_{50} value of 80 nM, and a K_d value of 230 nM was determined for the interaction of group IIA $sPLA_2$ with FXa[128]. These values appear consistent with a potential localized and/or systemic anticoagulant role of group IIA $sPLA_2$ during various physiological or pathological states. Hydrolysis of platelet-derived microvesicles by group IIA $sPLA_2$ (described above) could lead to an anticoagulant effect since these microvesicles support the coagulation cascade in addition to the platelet membrane[113]. However, the catalytically inactive mutant showed the same efficiency as wild-type group IIA $sPLA_2$ to inhibit thrombin generation in PRP, indicating that such phospholipid hydrolysis probably does not play a major role in the anticoagulant effect of this enzyme. Finally, group IIA $sPLA_2$ has been shown to increase the generation of prostacyclin by vascular endothelial cells[103]. This eicosanoid is an efficient inhibitor of platelet activation. The occurrence of such an effect in vivo would amplify the anticoagulant effect of group IIA $sPLA_2$.

Degradation of apoptotic and injured cells by group IIA $sPLA_2$

A membrane rearrangement that could lead to increased hydrolysis by the group IIA $sPLA_2$ is the appearance of phosphatidylserine during the apoptotic process in eukaryotic cells that is correlated with the loss of the membrane asymmetry and with membrane blebbing[130]. Cells undergoing apoptosis are sensitive to the action of the group IIA $sPLA_2$[131,132]. Therefore, one possible function of group IIA $sPLA_2$ released from either platelets or other cells is to eliminate apoptotic cells or injured cells[133]. This may constitute an important role in the removal of the hemostatic clot including activated platelets and microvesicles in order to avoid establishment of a prothrombotic situation.

Role of group IIA $sPLA_2$ in atherogenesis: low density lipoprotein (LDL) hydrolysis

Atherosclerosis is a disease involving the immune system and chronic inflammation in the initiation of endothelial cell dysfunction and the development of advanced lesions[134]. In addition to endothelial cells, macrophages, T-lymphocytes and smooth muscle cells collaborate to induce such a pathological situation. The 'CD40 receptor/CD40 ligand' system, a key mediator of cell communication in the immune system, is crucial in the establishment of atherosclerotic lesions[134]. The possible involvement of blood platelets in such a process has been pointed out since the CD40 ligand is expressed on their surface upon activation, and oxidatively modified LDL (low density lipoprotein) stimulates platelets[135]. The participation of the group IIA $sPLA_2$ in atherogenesis has been recently examined since it is expressed by cells implied in this process[136,137] and detected in human atherosclerotic plaques[138]. It has been shown that group IIA $sPLA_2$ is able to hydrolyse plasma lipoproteins and in particular mildly oxidized or aged LDL leading to modified LDL with higher affinity for proteoglycans and therefore higher atherogenic properties[139,140]. The group IIA $sPLA_2$ binds to heparan sulfate proteoglycans via their glycosaminoglycan moiety and to decorin, a small proteoglycan of the collagen network in atherosclerotic plaques, via the protein core[141]. Indeed, both LDL and group IIA $sPLA_2$ are likely to be colocalized at inflammatory loci via the binding to proteoglycans present on cell membranes and on the extracellular matrix of the arterial wall. Transgenic mice overexpressing group IIA $sPLA_2$ show increased atherogenesis and altered lipoproteins, supporting a role of this enzyme in atherosclerosis[142]. However, the lipoprotein-associated PLA_2 (Lp–PLA_2) which is found predominantly on LDL is also important for the modification of the LDL[143]. The exact function of Lp–PLA_2 vs. group IIA $sPLA_2$ in the establishment of atherosclerosis has still to be further investigated.

Antimicrobial effect of group IIA $sPLA_2$

It has been established that group IIA $sPLA_2$ contributes to the antimicrobial action in response to invading microorganisms[20,144,145]. Phagocytosis and consecutive bacterial membrane degradation by polymorphonuclear leukocytes represent an essential first-line defence against invading bacteria. Group IIA $sPLA_2$ participates in intracellular bacterial digestion by associating with the surfaces of bacteria and neutrophils before phagocytosis and acting after cointernalization with ingested bacteria. Group IIA $sPLA_2$ is also able to efficiently bind and extracellularly hydrolyse the membrane of gram positive bacteria[144]. In order to achieve such phospholipid hydrolysis from gram negative bacteria, group IIA $sPLA_2$ requires the presence of the BPI (bactericidal/permeability-increasing protein) that is released by polymorphonuclear leukocytes[146]. Transgenic mice with the group IIA $sPLA_2$ gene were reported to be more resistant to *Staphyloccocus aureus* and *Escherichia*

coli infection than control mice[120,147]. This antimicrobial action is likely to take place in the intestine where group IIA $sPLA_2$ is highly expressed in Paneth cells[148] and in tears[18]. During skin injury often associated with inflammation and thrombosis, it is possible that group IIA $sPLA_2$ secreted either from platelets or inflammatory cells also contribute to eliminate invasive bacteria.

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