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Molecular Determinants of Bacterial Sensitivity and Resistance to Mammalian Group IIA Phospholipase A2

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Abstract

Group IIA secretory phospholipase A_2 (sPLA$_2$-IIA) of mammalian species is unique among the many structurally and functionally related mammalian sPLA$_2$ in their high net positive charge and potent (nM) antibacterial activity. Toward the Gram-positive bacteria tested thus far, the global cationic properties of sPLA$_2$-IIA are necessary for optimal binding to intact bacteria and penetration of the multi-layered thick cell wall, but not for the degradation of membrane phospholipids that is essential for bacterial killing. Various Gram-positive bacterial species can differ as much as 1000-fold in sPLA$_2$-IIA sensitivity despite similar intrinsic enzymatic activity of sPLA$_2$-IIA toward the membrane phospholipids of the various bacteria. D-alanylation of wall- and lipo-teichoic acids in *Stapylococcus* aureus and sortase function in *Streptococcus pyogenes* increase bacterial resistance to sPLA$_2$-IIA by up to 100-fold apparently by affecting translocation of bound sPLA$_2$-IIA to the cell membrane. Action of the sPLA$_2$-IIA and other related sPLA$_2$ against Gram-negative bacteria is more dependent on cationic properties of the enzyme near the amino-terminus of the protein and collaboration with other host defense proteins that produce alterations of the unique Gram-negative bacterial outer membrane that normally represents a barrier to sPLA$_2$-IIA action.

Introduction

Acute inflammatory responses of mammalian hosts to invading bacteria include recruitment of polymorphonuclear leukocyte neutrophils from the bloodstream and mobilization of extracellular antimicrobial compounds that may originate from a variety of intravascular and extravascular sources (1–3). The latter often includes a secretory (Group IIA phospholipase A2 (sPLA$_2$-IIA)) that can function both independently against many Gram-positive bacteria and in concert with other host defense systems toward both Gram-negative and Gram-positive bacteria to increase digestion and killing of these bacteria (1, 2, 4–14). The sPLA$_2$-

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IIA is a member of a family of low M_r (14,000–19,000) secretory PLA2 with close overall structural and functional (enzymatic) similarity to related enzymes present in the venoms of snakes and insects and also in plants (15). Hallmarks of these proteins include a highly compact three-dimensional structure that is stabilized by 6–8 disulfide bonds and a calcium-dependent catalytic machinery mediating stereospecific hydrolysis of glycerophospholipids at the *sn*-2 position yielding free fatty acids and 1-acyl-lyso-phospholipids. Among the ten different secretory $sPLA_2$ expressed by humans, however, there are marked differences in the regulation of expression, sites of extracellular mobilization, and preferred biological targets consistent with distinct physiological roles of the various $sPLA_2$. The $sPLA_2$-IIA is unique in its very high net charge (up to +17) and antibacterial potency. At nM concentrations, the $sPLA_2$-IIA can attack both Gram-positive and Gram-negative bacteria, though the latter typically requires the assistance of other host defense proteins to facilitate access of the $sPLA_2$ to phospholipids in the Gram-negative bacterial envelope (Fig. 1). In contrast, host cells are highly resistant to $sPLA_2$-IIA, at least under normal resting conditions, as are viruses whose envelopes are derived from host cell membranes. Together, the unique antibacterial properties of the $sPLA_2$-IIA and its target cell selectivity suggest specific molecular and structural determinants of the antibacterial action of $sPLA_2$-IIA. This review will mostly focus on those properties that influence the action of this enzyme on Gram-positive bacteria.

Actions of purified $sPLA_2$-IIA against Gram-positive bacteria

Testing of the antimicrobial spectrum of $sPLA_2$-IIA has not been exhaustive but sufficient to reveal the susceptibility of many different Gram-positive bacterial species to doses of extracellular $sPLA_2$-IIA achievable in certain settings *in vivo* (11, 12, 16–19). *Bacillus subtilis* is among the most sensitive to $sPLA_2$-IIA, with as little as 0.1–1 nM (1.5–15 ng/ml) of human $sPLA_2$-IIA sufficient to produce 1–3 logs killing of 10^6 bacteria/ml within 1 hour of incubation. Similar effects on *S. aureus* and *Streptococcus agalactiae* (20) (Group B streptococci; GBS) generally (but see below) require 10–100× higher sPLA2-IIA concentrations. Other Gram-positive bacterial species (e.g., *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Enterococcus faecalis*) can also be killed by $sPLA_2$-IIA but require even higher (10–100-fold) doses (17, 20). Where studied, differences in bacterial sensitivity to the bactericidal effects of $sPLA_2$-IIA parallel differences in $sPLA_2$-IIA -triggered bacterial phospholipid (PL) degradation (17, 21, 22). A point mutation (D48S) in recombinant human $sPLA_2$-IIA that disrupts Ca^{2+} binding needed for catalytic activity ablates both $sPLA_2$-IIA -induced bacterial PL degradation and killing (9, 13). Bacterial killing requires rapid and massive PL degradation (>50% of total membrane PL within 30 min) followed almost immediately by loss of the PL degradation products (free fatty acids and lyso-PL) from the bacterial membrane to extracellular albumin (21, 23–24). The ability of bacteria treated with sub-lethal doses of $sPLA_2$-IIA (or otherwise lethal $sPLA_2$-IIA doses in the absence of albumin) to retain viability despite substantial degradation of membrane PL suggests a capacity of these treated bacteria to replace degraded and lost membrane PL by either *de novo* synthesis of PL or (in the absence of albumin) recycling of PL breakdown products that remain within the bacterial membrane (24). The mechanism(s) of this reparative process and its possible role in bacterial resistance to $sPLA_2$-IIA -mediated killing

deserve further study. An autolysin-deficient mutant strain of *S. aureus* is as sensitive as the parent strain to the phospho-lipolytic activity of the $sPLA_2$-IIA but requires several-fold higher $sPLA_2$-IIA doses to be killed (23). One possible interpretation of this finding is that rapid and extensive loss of membrane phospholipids leads to premature/inappropriate activation of autolysins that help convert potentially reversible membrane phospholipid loss to irreversible cell wall damage.

Whereas differences in the bactericidal potency of $sPLA_2$-IIA toward different species of Gram-positive bacteria correlate closely with different dose requirements to produce membrane PL degradation in intact bacteria, these differences do not reflect different substrate properties of the PL of these bacterial species, as judged either by assay of extracted PL presented as dispersions in aqueous solution or of cell wall-depleted protoplasts in which PL are presented as integral components of an intact cytoplasmic membrane (21, 22). Thus, the differences in sensitivity that are observed in various Gram-positive bacteria are a specific feature of the interactions of the intact bacteria with $sPLA_2$-IIA and hence, by implication, distinguishing properties of the cell wall that is assembled outside of the bacterial membrane.

The most revealing insights concerning the molecular and structural determinants of the antibacterial action of $sPLA_2$-IIA toward Gram-positive bacteria have been obtained in a series of studies of wild-type (wt) and mutant $sPLA_2$-IIA and wt and mutant *S. aureus* SA113). As indicated, the most striking physical feature of the $sPLA_2$-IIA is its remarkably high net positive charge that is manifest on virtually every exposed surface of the protein (Fig. 2). Mutational analyses of the human $sPLA_2$-IIA have confirmed the key role of this property in its activity against both *B. subtilis* and *S. aureus* (Table 1) (21, 25). Stepwise decrements in the net (+) charge of human $sPLA_2$-IIA achieved by substitution of basic amino acids with acidic residues produced a stepwise reduction in bactericidal potency toward both *S. aureus* and *B. subtilis*. Mutations at different sites of the enzyme had very similar effects on $sPLA_2$-IIA antibacterial potency, demonstrating the importance of the global cationicity of the $sPLA_2$-IIA (Fig. 2) in its antibacterial action. Activity toward *S. aureus* (vs. *B. subtilis*) appears to be somewhat more dependent on the very high net (+) charge of the sPLA2-IIA, as manifest both by the greater reduction in potency accompanying diminution of net charge from +15 to +13 and, conversely, the increase in potency accompanying an increase in net charge for +15 to + 17 (Table 1); (26).

This dependence of the bactericidal potency of the $sPLA_2$-IIA on its global charge properties parallels changes in PLA2 dose requirements for producing bacterial membrane PL degradation and loss. Remarkably, reduction of the net (+) charge of the $sPLA_2$-IIA by as much as 10 charge units (+15 to +5) does not reduce sPLA2-IIA activity toward the PL of *B. subtilis* (Table 1) or *S. aureus* when the PL are presented as part of cell wall-denuded membrane protoplasts (21). These findings suggest strongly that the unique cationic properties of the $sPLA_2$-IIA are important for initial (surface) interaction of the enzyme with intact Gram-positive bacteria and/or penetration of the enzyme through the cell wall but not for its interaction with and degradation of membrane PL once it has accessed the bacterial cytoplasmic membrane.

Appraising the effects of specific molecular and structural variables on initial $sPLA_2$-IIA interactions with the Gram-positive bacterial surface and underlying cell wall could be confounded by cell wall alterations that are induced secondary to massive degradation and loss of membrane PL (23). To preclude this possibility, initial $sPLA_2$-IIA binding has been measured in the absence of calcium to prevent calcium-dependent membrane PL degradation. That initial $sPLA_2$-IIA binding was calcium-independent was established by demonstrating equal binding of catalytically inactive D48S human $sPLA_2$-IIA in both the presence and absence of added 1 mM calcium chloride. Comparison of wt and charge variant [^{125}I]PLA2 binding to *S. aureus* showed stepwise alterations in $sPLA_2$-IIA binding that correlate with the net (+) charge of the enzyme (Fig. 3). However, effects of $sPLA_2$-IIA net charge on binding are significantly less than effects on antibacterial potency (compare Table 1 and Figure 3), suggesting additional effects of the cationicity of $sPLA_2$-IIA on cell wall penetration. To more directly test this hypothesis, doses of wt or charge variant $sPLA_2$-IIA present during the initial incubation of $sPLA_2$-IIA + *S. aureus* without calcium was adjusted to achieve roughly equal binding of each $sPLA_2$-IIA species. When the bacteria were washed to remove unbound PLA2 and incubated with calcium, the antibacterial effects of bound $sPLA_2$-IIA clearly correlated with the net charge of the bound enzyme (+15 > +13 > +11). In sum, the highly cationic properties of the sPLA2-IIA promote its antibacterial potency toward Gram-positive bacteria both by promoting initial surface binding and by increasing the efficiency of cell wall penetration of the bound enzyme to access membrane PL (Fig. 4). These charge-dependent non-catalytic interactions of the enzyme with the Gram-positive bacterial envelope make possible the calcium-dependent catalytic degradation of membrane PL that is required for bacterial killing. It is the ability of the wt $sPLA_2$-IIA to access the membrane PL of intact Gram-positive bacteria and not its ability to act on the membrane PL once exposed that differentiates this sPLA2 from other $sPLA_2$ and depends on its unique cationic properties.

Unpublished observations of ours have revealed only slightly greater calciumin-dependent $sPLA_2$-IIA binding to *B. subtilis* than to *S. aureus*, strongly suggesting that the remarkable sensitivity of *B. subtilis* to wt $sPLA_2$-IIA corresponds to much more facile penetration of the cell wall by $sPLA_2$-IIA in these bacteria.

Complementing the insights gained from the mutational and mechanistic studies of $sPLA_2$-IIA have been studies of the PLA2 sensitivity of a variety of envelope mutants of *S. aureus* derived from SA113. As charge modification of the $sPLA_2$-IIA has little impact on its ability to degrade membrane PL once the enzyme has access to the bacterial cytoplasmic membrane, charge modification of phosphatidylglycerol (PG), the major PL species of many Gram-positive bacteria including *S. aureus* and *B. subtilis*, has little effect on the sensitivity to $sPLA_2$-IIA of either intact *S. aureus* or cell-wall depleted membrane protoplasts (21). This has been demonstrated by comparing the sensitivity of wt and *mprF S. aureus*. MprF is a M_r 97,000 integral membrane protein that mediates: 1) modification of membrane PG with the cationic amino acid lysine at the cytoplasmic surface of the cytoplasmic membrane to convert PG to lysyl PG; and 2) translocation of the newly formed lysyl-PG to the outer leaflet of the membrane (27); i.e., the membrane leaflet that is accessed by sPLA2 (28). Whereas conversion of PG to lysyl PG has been linked to increased bacterial (e.g., *S.*

aureus) resistance to several different relatively small cationic antimicrobial peptides (CAMPs; 29) and also to daptomycin (30), *mprF* and wt *S. aureus* show little difference in sensitivity to $sPLA_2$-IIA and derived membrane protoplasts are virtually identical in sensitivity to $sPLA_2$-IIA (21).

In marked contrast, D-alanylation of cell envelope teichoic acids (TA), both peptidoglycan-linked wall teichoic acids (WTA) and membrane-inserted lipoteichoic acids (LTA) has a profound impact on the sensitivity of *S. aureus* (SA113) (intact bacteria but not membrane protoplasts) to $sPLA_2$-IIA (14, 21). *DltA S. aureus* are nearly 100-fold more sensitive to both the phospho-lipolytic and bactericidal actions of $sPLA_2$-IIA, in comparison to the wt parent strain. Together with the abundant peptidoglycan polymers that make up the matrix of the cell wall, WTA and LTA represent the major determinants of the net (-) charge of the Gram-positive bacterial surface and cell wall (31). In *S. aureus*, the WTA and LTA polymers are comprised of repeating units of ribitol (WTA) or glycerol (LTA) phosphate (32, 33). Together, WTA and LTA form a negatively charged lattice bridging the cell membrane through the cell wall (31). As such, it is conceivable that they provide an anionic "ladder" down which the highly cationic $sPLA_2$-IIA could advance, displacing as it proceeds less cationic autolysins that are normally constrained by electrostatic interactions with WTA and LTA (31, 34). Substitution of WTA and LTA by D-alanine leaves unshielded the (partial) positive charge of the free α-NH2 group of D-alanine and thus partially reduces the net (−) charge of the substituted WTA and LTA polymers (31, 35). The reduced sensitivity of wt vs. *dltA S. aureus* to a variety of small CAMPs including Magainin II amide and human β-defensin 3(HBD-3) has been attributed to the charge-neutralizing effects of D-alanine substitution (36). However, these lead to only 3-fold changes in sensitivity of wt and *dltA S. aureus* under the same experimental conditions in which sensitivity to $sPLA_2$-IIA is changed 100-fold (21). Similar comparative analyses of sPLA2 binding, cell wall penetration, and activity as described above for wt and mutant $sPLA_2$-IIA revealed no effect of D-alanylation of TA on either initial calcium-independent sPLA2-IIA binding (Figure 5) or activity against membrane protoplasts (21). Remarkably, the marked increase in sensitivity of *dltA S. aureus* was equally manifest with wt and mutant human $sPLA_2$-IIA of lower net (+) charge (+13 or +11 vs. +15 of wt enzyme) (21). These findings suggest that the principal effect of D-alanylation of TA (WTA and LTA) vis a vis $sPLA_2$-IIA action against *S. aureus* is on penetration of bound PLA2 to the cell membrane. That this effect of D-alanylation is manifest irrespective of the net charge of the sPLA2-IIA may mean that the effect of D-alanine substitution is more a consequence of steric hindrance of cell wall penetration caused by the bulkier presence of the substituted D-alanine residue (vs. associated divalent cations) than one based on reduced electrostatic interactions although other possible pleiotropic consequences of altered dltA function can not be excluded. It should be emphasized that despite the far greater effect of D-alanylation of TA on the potency $sPLA_2$-IIA (vs. CAMPs) toward wt *S. aureus*, the molar potency of $sPLA_2$-IIA toward wt *S. aureus* is still nearly 100× greater than that of the various non-catalytic CAMPs. Whether or not this reflects the greater efficiency, even in wt *S. aureus*, of binding and cell wall penetration of the wt $sPLA_2$-IIA (vs. CAMPs) or the ability of even a very small number of molecules of $sPLA_2$-IIA reaching the bacterial cytoplasmic membrane to produce lethal damage by virtue of its catalytic properties is not yet known.

Several other genotypic and phenotypic modifiers of sPLA2-IIA potency toward *S. aureus* have been identified that support the notion that the efficiency of cell wall penetration is a key determinant of $sPLA_2$-IIA potency. This includes the greater sensitivity of: 1) logarithmic vs. stationary phase bacteria (or bacteria pre-treated with a bacteriostatic antibiotic) (23); 2) bacteria pre-treated with a sub-inhibitory dose of a β-lactam antibiotic to reduce cell wall peptidoglycan cross-linking (23); and 3) wt vs. *tag O S. aureus* (lacking WTA) (22). In each instance, initial PLA2 binding and activity vs. isolated membrane protoplasts are essentially the same, strongly suggesting selective effects of these bacterial modifications on $sPLA_2$-IIA cell wall penetration. Bound PLA2 could be nearly fully displaced from the bacteria with 1M NaCl, confirming that in each instance bacterial binding of PLA2 was mediated by initial electrostatic interactions between the cationic protein and anionic sites exposed on the bacterial surface. $sPLA_2$-IIA binding to *tagO S. aureus* demonstrated unequivocally that this binding was not dependent on interactions with WTA, a somewhat surprising result. However, exchange of substituted D-alanine between WTA and LTA have suggested close physical proximity of these abundant polyanionic polymers (31, 37; Figure 1). If so, initial $sPLA_2$-IIA binding to WTA may provide the most favorable route for translocation of bound PLA2 from the bacterial surface to the cell membrane. This migration of bound PLA2 may – en route – lead to localized displacement of autolysin(s) bound to TA, promoting localized autolysin activity (e.g., severing of peptidoglycan cross-links) and more efficient $sPLA_2$-IIA penetration of the cell wall.

Similar detailed studies have not yet been carried out in other Gram-negative bacterial species leaving open the question of whether or not similar mechanistic concepts apply to $sPLA_2$-IIA action against other Gram-positive bacteria. An important exception has been provided by recent studies seeking to better understand the basis of the generally much lower sensitivity of Group A vs. Group B streptococci to $sPLA_2$-IIA (20). These studies have revealed an important role of proteins covalently tethered to the cell wall via specific linkages dependent on sortase A. Increased sensitivity of *SrtA S. pyogenes* to the bactericidal action of $sPLA_2$-IIA was paralleled by increased $sPLA_2$-IIA -triggered bacterial PL degradation despite greater PLA2 binding to wt bacteria. These findings suggest that in *S. pyogenes* (Group A streptococci), sortase A-dependent cell wall proteins provide a significant impediment to the access of bacterial bound PLA2 to the bacterial cytoplasmic membrane.

Actions of $sPLA_2$-IIA and other related $sPLA_2$ against Gram-negative bacteria

In contrast to the ability of nM concentrations of purified $sPLA_2$-IIA to act against a variety of Gram-positive bacteria, independent antibacterial activity of sPLA toward the Gram-negative bacteria thus far tested including *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Neiseria meningitidis* requires enzyme concnetrations that greatly exceed $sPLA_2$-IIA levels at most or perhaps all body sites even during inflammation (9, 38). However, redistribution of phospholipids from the inner to outer leaflet of the Gram-negative bacterial outer membrane (Fig. 1), as likely induced by sublethal actions of the neutrophil bactericidal/permeability-increasing protein (BPI) and the complement

membrane-attack complex (1, 10) or during shedding of outer membrane vesicles ("blebs"; 39) render some of bacterial phospholipids susceptible to $sPLA_2$-IIA. The rate and extent of membrane phospholipid degradation by $sPLA_2$-IIA in concert with BPI or the membrane-attack complex depends on the extent of envelope alterations produced by BPI and the membrane-attack complex and the concentration of $sPLA_2$-IIA. These requirements for $sPLA_2$-IIA action toward the Gram-negative bacteria tested to date are consistent with the restricted access of $sPLA_2$ to phospholipids in the outer leaflet of an intact bilayer structure (28) and the asymmetry in distribution of lipopolysaccharides (LPS) and phospholipids in the outer membrane of these bacteria (Fig. 1; 40). The extent to which these properties apply to other Gram-negative bacteria requires further study.

The activity of $sPLA_2$-IIA toward BPI- or membrane-attack complex-treated Gram-negative bacteria (e.g., *Escherichia coli*) is also dependent on the cationic properties of these enzymes that promote non-catalytic interactions of the enzyme with the altered bacterial surface (41, 42). However, in contrast to the requirements for $sPLA_2$ action against Gram-positive bacteria, certain functionally related $sPLA_2$ with much lower global cationic properties (e.g., basic $sPLA_2$ isoform pf *Agkistrodon halys blomhoffii* venom (net charge of +7); human pancreatic $sPLA_2$-IB (net charge of +3) that have <0.1% the activity of human $sPLA_2$-IIA toward *S. aureus* and *B. subtilis* (Liang NS and Weiss JP; unpublished observations) display substantial activity toward BPI-treated *E. coli* (41, 42). For those two $sPLA_2$, BPI-dependent binding and degradation of phospholipids of *E. coli* depend on a cluster of basic residues along the polar face of an alpha-helix near the NH_2-terminus that represents a variable surface region among all $sPLA_2$ (42, 43). Mutational analyses in recombinant human $sPLA_2$-IIA also support a key role of basic amino acids in this region in the action of the $sPLA_2$-IIA on BPI- and complement-treated *E. coli*, as well as on *E. coli* ingested by neutrophils (9).

Antibacterial actions of $sPLA_2$-IIA in biological fluids, *ex vivo*

$sPLA_2$-IIA -rich biological fluids, both those that are constitutively $sPLA_2$-IIA -rich (e.g., tear fluid, seminal plasma; 16, 44) and those in which the presence of $sPLA_2$-IIA is triggered by either sterile or infectious inflammation (6, 11, 12, 18, 45), display potent antibacterial activity against *Staphylococcus aureus* and a number of other Grampositive bacterial species. $sPLA_2$-IIA levels in inflammatory fluids can be as high as 500 nM and in tear fluids up to 5 μM, sufficient to account for most if not nearly all of the bactericidal activity of these fluids toward, for example, *S. aureus*. Blood plasma and many tissue fluids under resting conditions, by contrast, contain <1 nM $sPLA_2$-IIA and no measurable activity toward the same Gram-positive bacteria. $sPLA_2$-IIA is fully active in fluids containing physiologic (extracellular) levels of monovalent and divalent cations.

Studies of $sPLA_2$-IIA-rich inflammatory fluids have been instrumental in showing the potential contribution of mobilized extracellular $sPLA_2$-IIA to digestion and disassembly of bacteria ingested by neutrophils and, in so doing, the integration of mobilized cellular and extracellular host defenses (1, 2, 9, 13, 14). This role has been demonstrated toward both Gram-negative (e.g., *Escherichia coli*) and Gram-positive (e.g., *S. aureus*) bacteria under biological conditions in which the extracellular $sPLA_2$-IIA alone or even together with the

whole inflammatory fluid produced much less bacterial PL degradation because of limiting levels of extracellular $sPLA_2$-IIA and/or the intrinsic resistance of the bacterial target. In each circumstance, the contribution of extracellular $sPLA_2$-IIA could not be substituted by other human $sPLA_2$, including those (Group V and Group X $sPLA_2$) present natively in the neutrophil (46), nor by mutants of $sPLA_2$-IIA that are not active when assayed alone vs. *S. aureus* or together with host defense proteins (e.g., neutrophil-derived bactericidal/permeability-increasing protein, membrane-attack complex of complement) that perturb the Gram-negative bacterial outer membrane, rendering Gram-negative bacteria more susceptible to the $sPLA_2$-IIA. Many more synergistic interactions involving the $sPLA_2$-IIA, for example with peptidoglycan-interacting and degrading proteins (e.g., lysozyme) present in both the cellular and extracellular compartments of neutrophil-rich exudates, seem likely and deserving much further study.

Concluding remarks

The defined nature of the biochemical action of the $sPLA_2$-IIA has made characterization of its specific interactions and actions on bacterial targets more amenable than for most antimicrobial compounds that, by contrast, exert their efforts by non-catalytic mechanisms that are often difficult to clearly distinguish one from the other. What also stands out in the antibacterial action of the $sPLA_2$-IIA is the integration of both initial non-catalytic interactions with the bacterial envelope that subsequently make possible the catalytic events (membrane phospholipid degradation) that are ultimately linked to bacterial injury and death. These distinct actions of the enzyme, all required for its potent antibacterial action, have made possible identification of different structural and functional attributes of the protein that are needed at different stages of the enzyme's antibacterial interactions. This, in turn, has provided an unusually well-defined context in which to determine the mechanisms by which specific bacterial properties and components affect sensitivity and resistance to the $sPLA_2$-IIA. Finally, by virtue of the specificity of the mechanistic insights gained, clearly different determinants of bacterial sensitivity and resistance to the PLA_2 vs. many of the smaller and non-catalytic cationic antimicrobial peptides have been able to be appreciated (47), an insight that should be exploited in future efforts for new drug development.

Supplementary Material

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Highlights

- Humans express ten closely related secreted phospholipases A_2 ($sPLA_2$)
- Unique features of Group IIA $sPLA_2$: strong cationicity; nM antibacterial activity
- Cationic properties of $sPLA_2$-IIA promote bacterial binding, cell wall penetration
- Bacterial death follows membrane phospholipid degradation, activation of autolysins
- Antibacterial actions of $sPLA_2$-IIA enhanced by other host defense systems

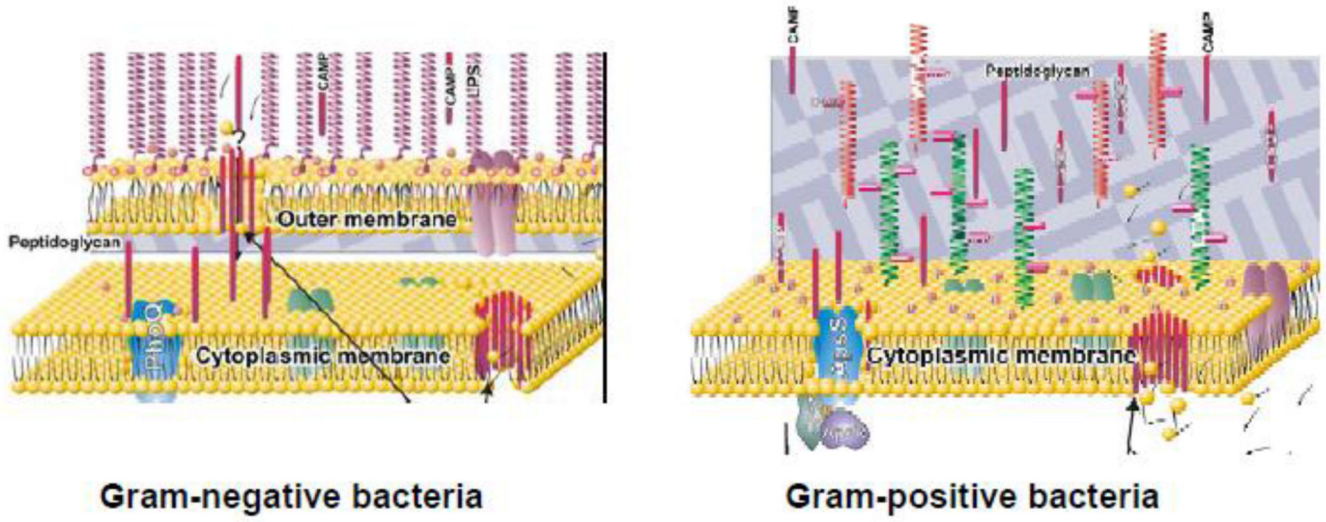

Figure 1.

Schematic structure of envelopes of Gram-negative (left) and Gram-positive (right) bacteria. Note the unique asymmetry to lipid arrangement in the outer membrane of Gram-negative bacteria, with lipopolysaccharides (LPS) occupying the outer leaflet and phospholipids mainly restricted to the inner leaflet. Figure is adapted from (47).

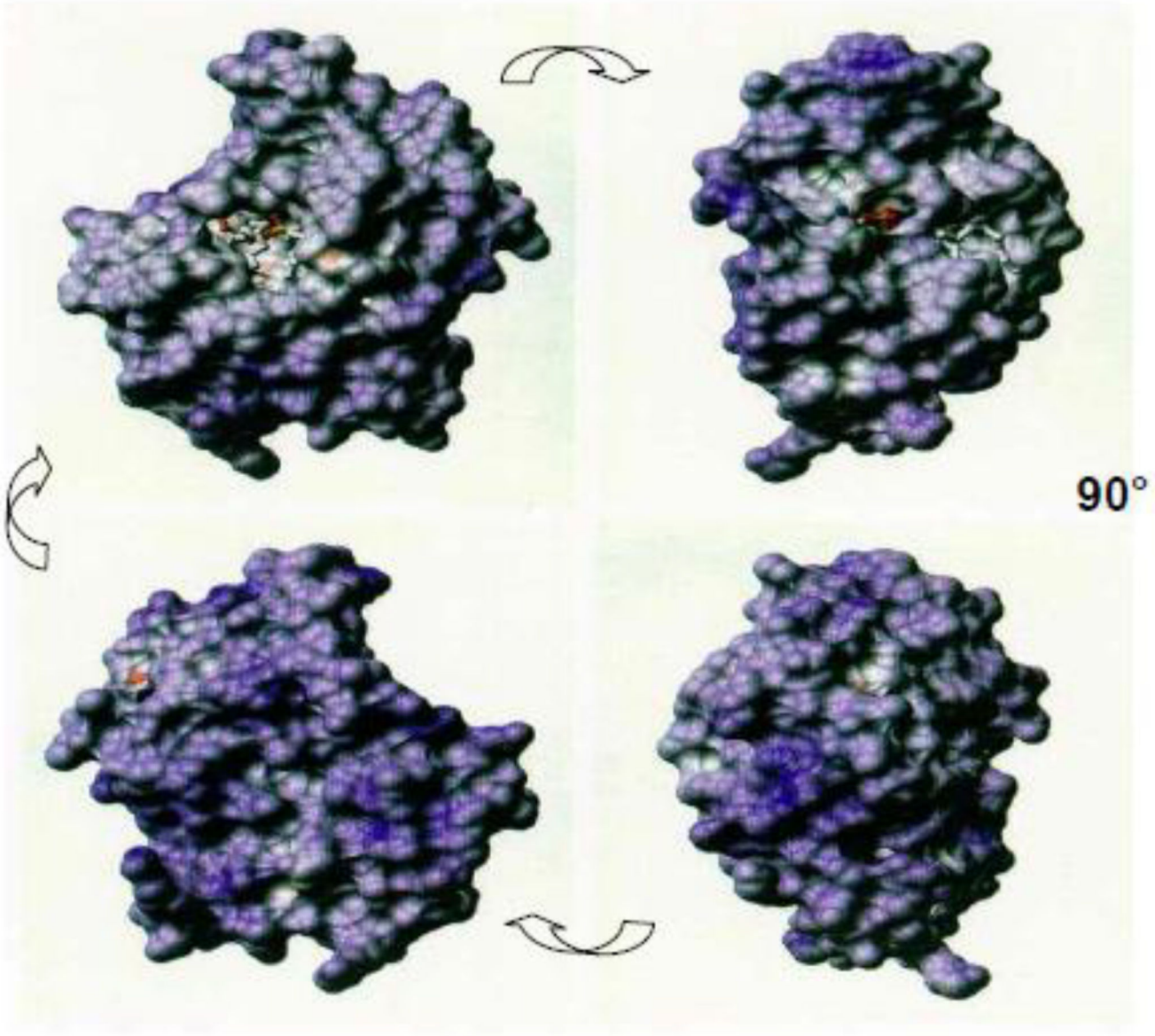

Figure 2.

Surface charge distribution in human $sPLA_2$-IIA. Cationic surfaces are represented in blue, with darker shades of blue signifying regions with higher net cationic character. Conversely, acidic surface regions are represented by shades of red. Note bound substrate analog in upper left image, just left and above center of that image. The four images were generated by 90° rotations, as indicated.

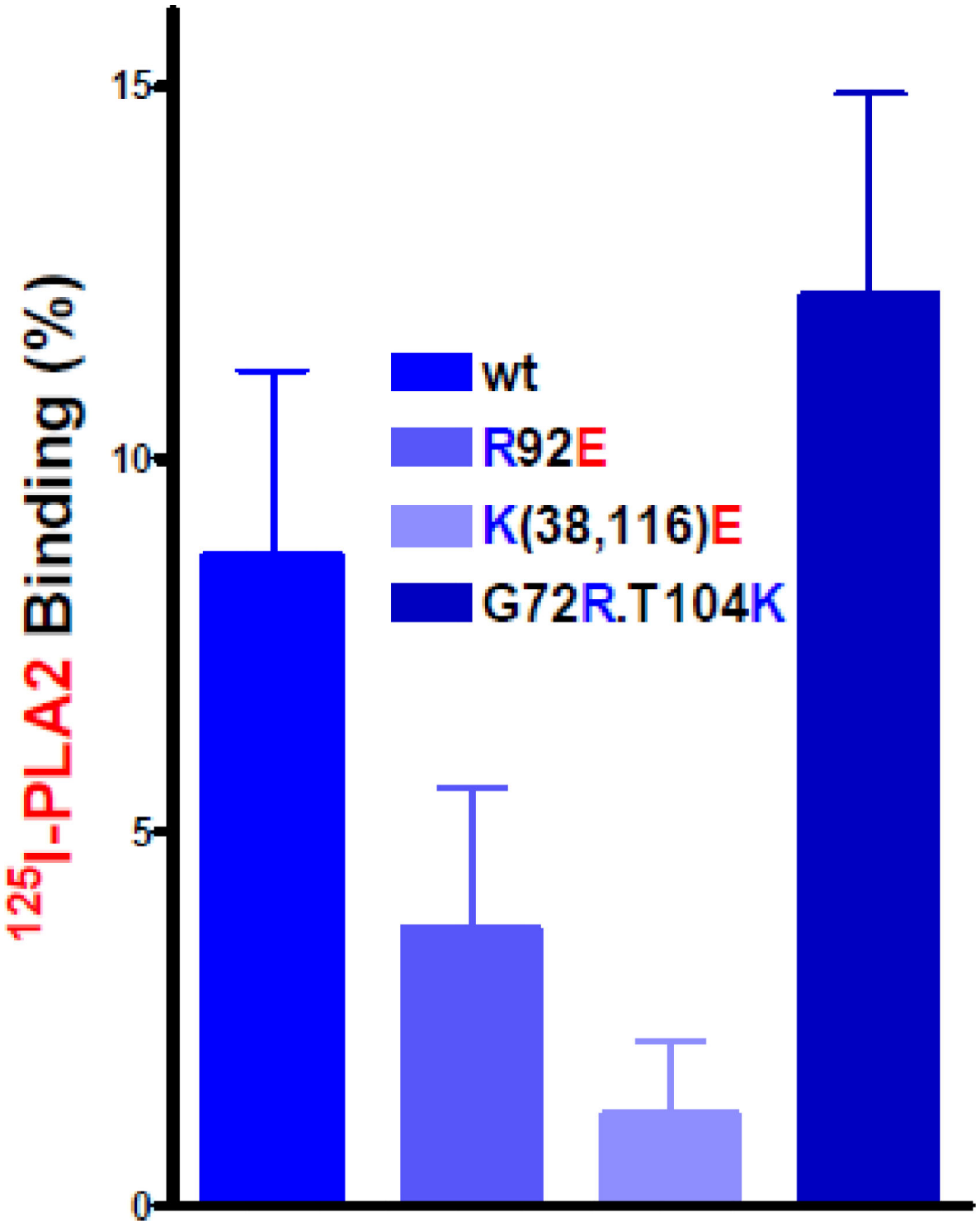

Figure 3.

Comparison of binding of [^{125}I] wt and charge variant recombinant human sPLA$_2$-IIA to wt *S. aureus* SA113. Incubations contained 5 × 10^6 bacteria and 50 ng sPLA$_2$-IIA in 1 ml of Hanks buffered salts solution without calcium and magnesium supplemented with 10 mM HEPES (pH 7.4) and 0.1% albumin. Results shown represent the mean ± SEM of three experiments, each done in duplicate.

- BINDING: **Yes** (Ca^{++}-independent)
- PENETRATION: **Yes**
- PL DEGRADATION: **No** (Ca^{++}-dependent)
- ACTIVATION OF AUTOLYSINS: **?**

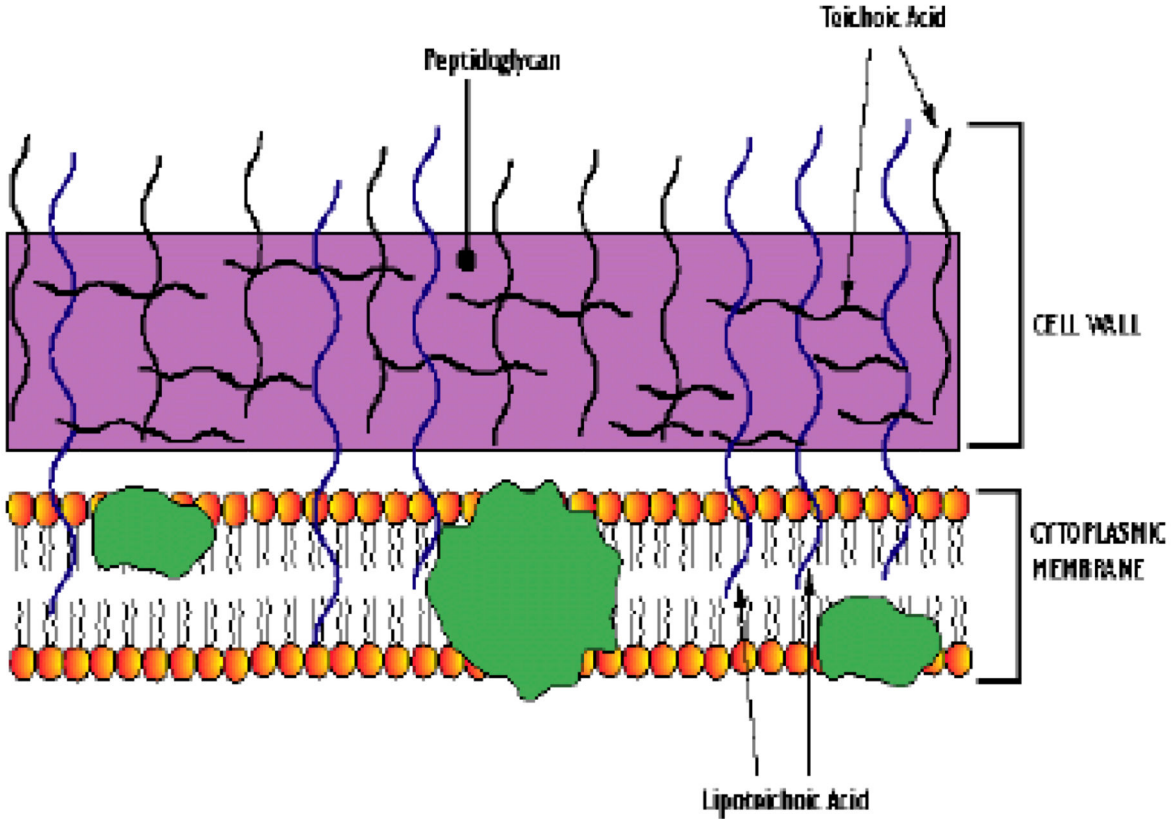

Figure 4.
Summary of required steps in bactericidal action of $sPLA_2$-IIA vs. Gram-positive bacteria (e.g., *S. aureus*) and role of cationic properties of the $sPLA_2$-IIA in each step of the process.

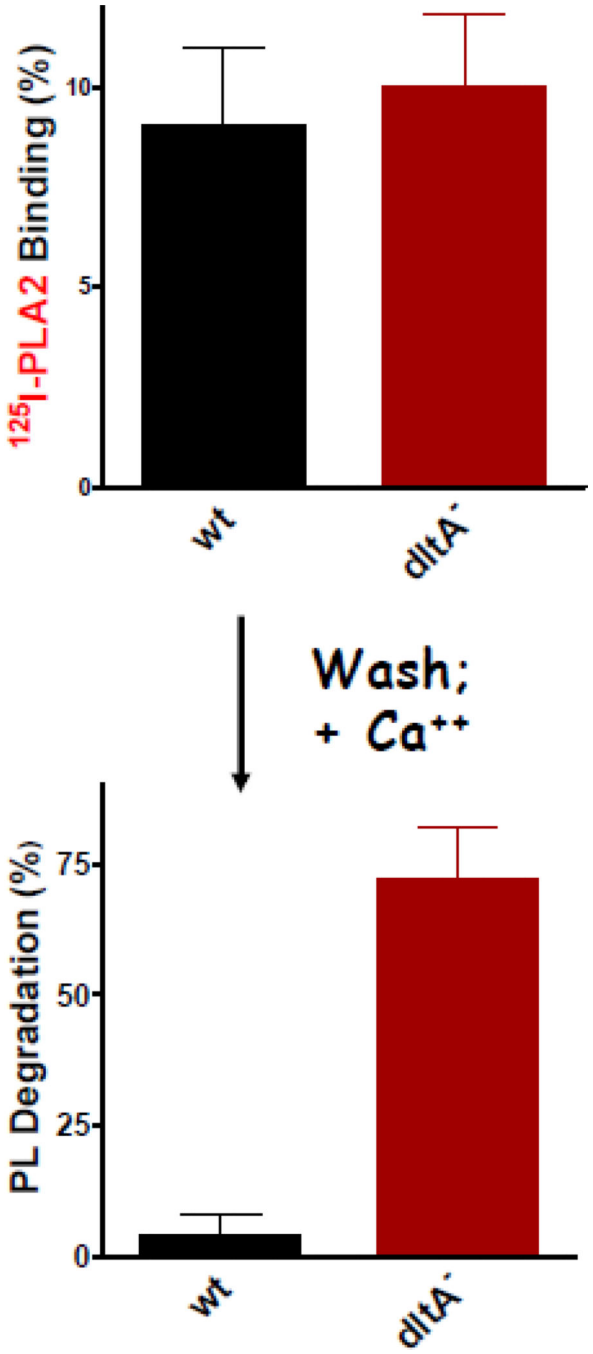

Figure 5.

D-alanylation of teichoic acids of *S. aureus* (SA113) markedly inhibits ability of bound human sPLA$_2$-IIA to degrade membrane PL of intact bacteria. Binding of [^{125}I] wt sPLA$_2$-IIA was measured after incubation of bacteria without calcium. Unbound PLA$_2$ was removed by washing and bacteria with bound PLA$_2$ was incubated with calcium to monitor calcium-dependent PL degradation. Initial incubations contained 5 × 10^6 bacteria and 1 ng

PLA_2 (lower dose of $sPLA_2$-IIA (vs. that use in experiments shown in Fig. 3) reflects exquisite sensitivity of the *dltA S. aureus*.

Table 1

Role of cationic properties of human $sPLA_2$-IIA in its activity toward intact Gram-positive bacteria and isolated membrane protoplasts.

		Activity (relative to wt $sPLA_2$-IIA;%)		
Recombinant Human $sPLA_2$-IIA**	**Net charge**	***S. aureus****	***B.subtilis****	***B.subtilis* protoplasts****
Wt	+15	100	100	100
K92E;K87E;K74E;B7E	+13	6±3	65±9	185±30
K110E.K115E; K38E.K116E; K10E.K16E;R7E;K16E;K124E.R127D	+11	0.4±0.3	4±3	146±45
K74E.K87E.R92E;R7E;K10E;K16E	+9	<0.1	<0.1	135±50
K38E.K110E.K115E.K116E	+7	<0.1	<0.1	120
K53E.R54E.R58E.K124E.R127D	+5	<0.1	<0.1	170
G72K.T103K	+17	484±82	122±30	N.T.
Pig $sPLA_2$-1B	−1	<0.1	<0.1	100

* All activities were measured as dose required to produce 90% bacterial killing (**) or 30% PL degradation (***). See references (21, 25) for description of wt and mutant enzymes and functional assays.

**** Mutant recombinant human $sPLA_2$-IIA were prepared by Dr. Ning-Sheng Liang (G72K.T103K) and Drs. R. Koduri and MH Gelb (all others). Data shown were collected by Dr. Liang and represent the mean ± SEM of at least three experiments.