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The Antibacterial Properties of Secreted Phospholipases A_2

A MAJOR PHYSIOLOGICAL ROLE FOR THE GROUP IIA ENZYME THAT DEPENDS ON THE VERY HIGH pI OF THE ENZYME TO ALLOW PENETRATION OF THE BACTERIAL CELL WALL*

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The antibacterial properties of human group IIA secreted phospholipase A_2 against Gram-positive bacteria as a result of membrane hydrolysis have been reported. Using *Micrococcus luteus* as a model system, we demonstrate the very high specificity of this human enzyme for such hydrolysis compared with the group IB, IIE, IIF, V, and X human secreted phospholipase A_2s. A unique feature of the group IIA enzyme is its very high pI due to a large excess of cationic residues on the enzyme surface. The importance of this global positive charge in bacterial cell membrane hydrolysis and bacterial killing has been examined using charge reversal mutagenesis. The global positive charge on the enzyme surface allows penetration through the bacterial cell wall, thus allowing access of this enzyme to the cell membrane. Reduced bacterial killing was associated with the loss of positive charge and reduced cell membrane hydrolysis. All mutants were highly effective in hydrolyzing the bacterial membrane of cells in which the cell wall was permeabilized with lysozyme. These same overall characteristics were also seen with suspensions of *Staphylococcus aureus* and *Listeria innocua*, where cell membrane hydrolysis and antibacterial activity of human group IIA enzyme was also lost as a result of charge reversal mutagenesis.

An increasing number of nonpancreatic $sPLA_2$s[1] have now been identified as a result of cloning strategies and have been reviewed (1). Although most information is available for the group IIA enzyme (reviewed in Ref. 2), structural and functional information is now accumulating for the group V and X enzymes (3, 4), while, more recently, the IID, IIE, IIF, and XII enzymes have been cloned and shown to have lipolytic activity (1, 5, 6). The diversity of primary sequence of these enzymes coupled with distinct tissue distribution profiles argues for distinct physiological roles for each mammalian $sPLA_2$ species.

The group IIA human $sPLA_2$ was first discovered in the synovial fluid of patients with rheumatoid arthritis and was also released from platelets (7, 8). The enzyme has a marked preference for anionic interfaces and expresses high activity with *Escherichia coli* membranes often used in standard assays, while a substrate preference for phosphatidylglycerol and phosphatidic acid has been proposed (9). The enzyme is now recognized as expressing antibacterial activity against Gram-positive bacteria, while its presence at high concentrations in inflammatory fluids (7) and human tears (10) is consistent with this activity as a major role of this phospholipase. Similarly, the enzyme is released from Paneth cells of the intestine (11) and macrophages (7, 8), both cells being involved in the human antibacterial response. It has been proposed that the antibacterial role of the group IIA enzyme is directly linked to the highly cationic nature of the protein, allowing bacterial cell wall penetration (12). The protein has a net tabulated charge of +19 (+27; −8), assuming cationic groups are His, Arg, and Lys and anionic groups are Asp and Glu (13).

The human group V and X enzymes have been identified more recently and their ability to hydrolyze mammalian cell membranes has been investigated (4, 14, 15); however, only the antibacterial properties of the group V enzyme have been described (16). These two enzymes show notable difference in tabulated positive charges of +10 (+18; −8) and −3 (+12; −15), respectively.

In this paper, we show using charge reversal mutagenesis that the unique ability of the human group IIA enzyme to penetrate the bacterial cell wall and to hydrolyze the cell membrane is attributable to the highly cationic nature of the enzyme. A loss of this hydrolyzing ability together with the loss of the antibacterial properties of the enzyme parallels removal of this positive charge. In all cases, bacterial killing paralleled the ability of the enzyme to achieve cell membrane hydrolysis; however, the expressed bactericidal properties were much less sensitive to enzyme activity, requiring prolonged exposure with higher concentrations of enzyme. It would appear that in the absence of other factors, these bacteria are able to recover from a considerable insult in terms of membrane phospholipid hydrolysis, as previously reported (17).

In contrast to the group IIA enzyme, we show that the human group V was very much less effective in its ability to penetrate the bacterial cell wall and hydrolyze the cell membrane although the group V enzyme has considerable cationic character. The group IB, IIE, IIF, and X human enzymes were also studied, and their characteristics are not consistent with them having an antibacterial role.

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[1] The abbreviations used are: $sPLA_2$, secreted phospholipase A_2; DOPG, dioleoylphosphatidylglycerol; HBSS, Hanks' balanced salts solution.

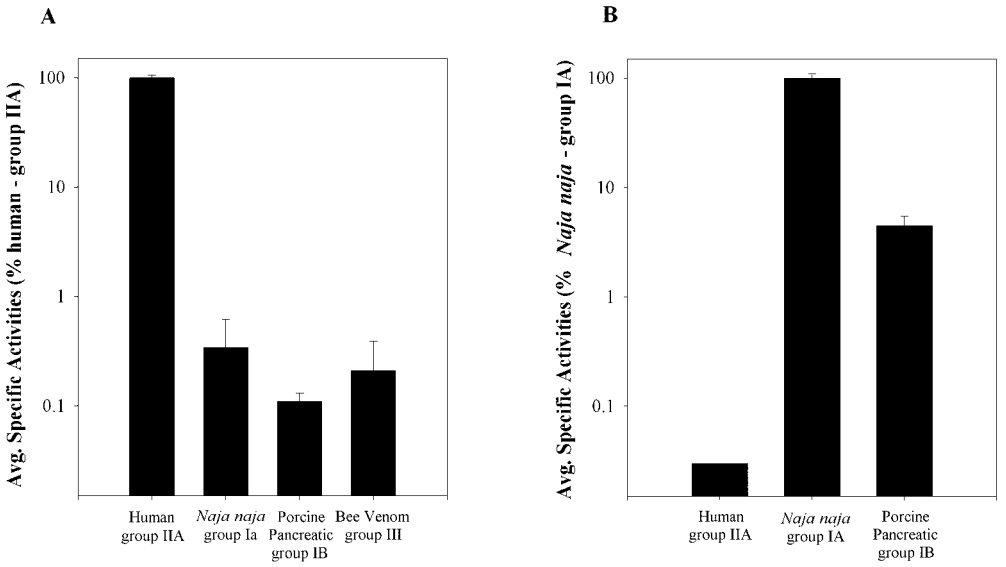


FIG. 1. **Initial rates of hydrolysis of bacterial (*A*) and eukaryotic (*B*) cell membranes.** Specific activities were determined for human group IIA, *N. naja* group IA, porcine pancreatic group IB, and bee venom group III $sPLA_2$s against *M. luteus* (2×10^7 cells/ml) (*A*) and L929 fibroblasts (4×10^5 cells/ml) (*B*) using a fluorescence displacement assay described under "Experimental Procedures." Data shown are means ± S.D. ($n = 3$).

EXPERIMENTAL PROCEDURES

Materials—Oleic acid, *Naja naja* and bee venom $sPLA_2$s, human milk lysozyme and lysostaphin from *Staphylococcus staphlolyticus* were obtained from Sigma. Porcine pancreatic $sPLA_2$ was purchased from Roche Molecular Biochemicals. The group IIa human $sPLA_2$ was prepared from a synthetic gene expressed in *E. coli* as a one-Ala (N1A) mutant and is essentially identical to the wild-type enzyme (9, 18). Charge reversal mutants of N1A were prepared as previously described (19, 20). The cloning and expression of the human group IB, IIE, IIF, V, and X enzymes has been described elsewhere (4, 21–23). 11-(dansylamino)-undecanoic acid was obtained from Molecular Probes, Inc. (Eugene, OR). DOPG was purchased from Avanti Polar Lipids (Alabaster, AL). Fatty acid-binding protein was prepared as previously described (24). The purity of all proteins was confirmed by SDS-PAGE.

Bacteria—M. luteus (9278) and *S. aureus* (12702) were obtained from NCIMB Ltd. (Aberdeen, UK). *Listeria innocua* (12210) were purchased from NCTC (London, UK). Bacteria were grown overnight at 37 °C, *M. luteus* in Luria Bertani medium and *S. aureus and L. innocua* in brain heart infusion broth. Bacteria were then diluted into fresh medium and grown further at 37 °C until an optical density (600 nm) of 0.45 was reached. Cells were harvested at $4000 \times g$ for 10 min, resuspended in HBSS, and kept on ice until ready for use.

Fluorescence Displacement PLA_2 Assays—The fluorescence displacement assay has been described previously (25). Briefly, stock assay buffer (20 ml) was prepared containing HBSS, 1 mM $CaCl_2$, and 1 μM 11-(dansylamino)-undecanoic acid. Assays were performed in stock assay buffer containing *M. luteus* at a concentration of $\sim 2 \times 10^7$ cells/ml and fatty acid-binding protein (~10 μg) at a final volume of 1 ml. Assays utilizing *Staphylococcus aureus* and *L. innocua* contained bacteria at the same optical density (600 nm) as for those with *M. luteus*. Assays using DOPG vesicles have been described (26). Assays were performed in plastic fluorometric cuvettes using a Hitachi F2500 fluorometer coupled to a computer for data recording. All assays were calibrated by the addition of known amounts of a methanol solution of oleic acid to a control assay containing all assay components except enzyme. All assays were performed at 37 °C.

Bactericidal Assays—Bacterial viability was assessed by measuring colony-forming ability of bacteria incubated in the absence or presence of PLA_2 for various times. Incubation mixtures contained $1–2 \times 10^7$ bacteria/ml in sterile HBSS supplemented with 1 mM sterile $CaCl_2$, 50 mg/ml sterile bovine serum albumin, and the appropriate amount of human $sPLA_2$. Reactions were carried out at 37 °C for up to 2 h. At various time points, aliquots were taken and serially diluted into sterile HBSS supplemented with 1 mM EGTA. 10-μl samples were then streaked onto media agar plates, and colony-forming units determined after 24–36 h of growth at 37 °C.

RESULTS

Comparison of the Bacterial Membrane-hydrolyzing Properties of Human Group IB, IIA, IIE, IIF, V, and X and Bee Venom PLA_2s—We have shown previously that the IIA enzyme is able to hydrolyze suspensions of *M. luteus*, whereas the enzymes from porcine pancreas and *N. naja* venom expressed very low activity with this substrate (27). This is in sharp contrast to the effect of these enzymes on mammalian cells, as illustrated in Fig. 1, where a log scale comparison between suspensions of *M. luteus* and L929 fibroblasts is shown. In particular, the very low activity of the *N. naja* venom enzyme on *M. luteus* as compared with high activity on L929 fibroblasts is highlighted, the complete reversal of the situation with the human group IIA enzyme. *M. luteus* was chosen because of its sensitivity to lysozyme, allowing an effective method for making the bacterial cell wall more permeable.

The highly variable ability of $sPLA_2$s to hydrolyze suspensions of *M. luteus* was extended to the study of a number of other human $sPLA_2$s, namely the IB, IIE, IIF, V, and X as shown in Fig. 2. It is clear that apart from the IIA enzyme, only the group V enzyme demonstrates significant ability to hydrolyze suspensions of intact *M. luteus*. This considerable difference in expressed activity with the different $sPLA_2$s must reflect the ability of the enzyme to penetrate the bacterial cell wall as prior treatment of the *M. luteus* with lysozyme allowed all enzymes to express enhanced activities (Fig. 2). We noted a variation in lysozyme response between different preparations of *M. luteus* seen as variable stimulation of hydrolysis by the $sPLA_2$s after lysozyme treatment. We attribute this to differences in the precise physiological state of the organism on harvesting that affects cell wall structure and hence sensitivity to lysozyme. These differences do not affect the conclusions, since relative sensitivity to hydrolysis between different $sPLA_2$s does not alter.

An explanation for the differential permeability of enzymes of essentially identical size is that the highly cationic nature of the IIA enzyme allows it to penetrate the highly anionic bacterial cell wall (27). In contrast, the group V enzyme, although cationic, has a net positive charge of +10 compared with +19 for the group IIA enzyme and manages a modest cell wall

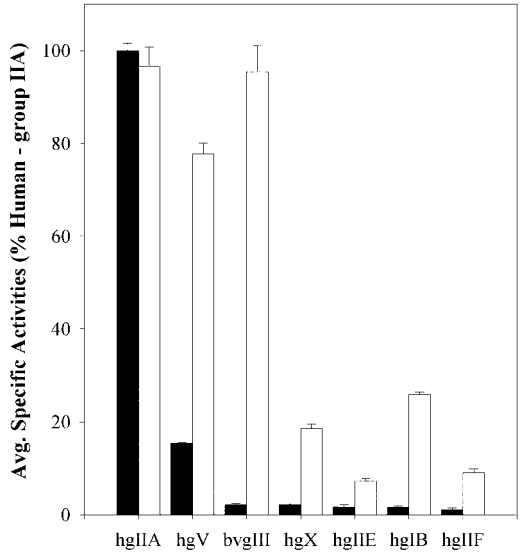
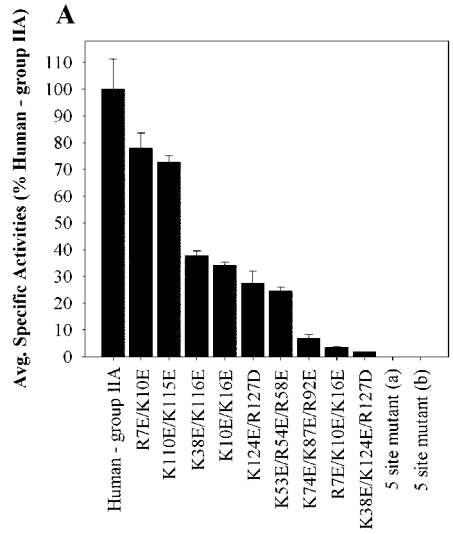


FIG. 2. **Activity of various $sPLA_2$s against *M. luteus* suspensions and the effect of lysozyme.** Specific activities were determined for human groups IIA (*hgIIA*), V (*hgV*), hgX (*hgX*), hgIIE (*hgIIE*), hgIB (*hgIB*), hgIIF (*hgIIF*), and bee venom group III (*bvgIII*) $sPLA_2$s (200 ng) against intact (*solid bars*) *M. luteus* (1×10^7 cells/ml)- and lysozyme (20 μg)-treated cells (*open bars*) using a fluorescence displacement assay described under "Experimental Procedures." Data shown are means ± S.D. ($n = 3$).



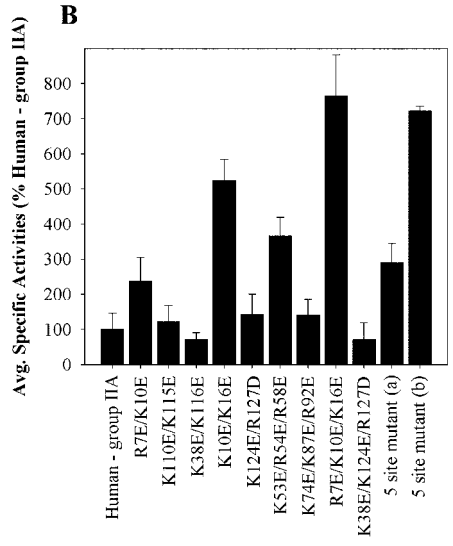


FIG. 3. **The ability of native and charge reversal mutants of human group IIA $sPLA_2$ to hydrolyze *M. luteus* membranes (*A*) and DOPG vesicles (*B*).** Specific activities were determined for native and charge reversal mutants of human group IIA $sPLA_2$ using a fluorescence displacement assay. Five-site mutant *a* was K53E/R54E/R58E/K124E/R127D, and five-site mutant *b* was R7E/K10E/K16E/K124E/R127D. Activities are expressed as percentage of that of the native enzyme against intact *M. luteus* (1×10^7 cells/ml) (*A*) and against DOPG small unilamellar vesicles (63 nmol/ml) (*B*) described under "Experimental Procedures." Data shown are means ± S.D. ($n = 3$).

penetration to allow a lower rate of cell membrane hydrolysis. The group X enzyme, which is inactive, is anionic with a net charge of −3. The IIE and IIF enzymes are also essentially inactive under these assay conditions and have tabulated net charges of +9 (+20; −11) and −6 (+14; −20), respectively, based on the total number of Lys, Arg, His, Asp, and Glu residues in the sequence (23). Recombinant human 1B pancreatic enzyme (21) with a net tabulated charge of +5 was also tested and showed no activity, behaving similarly to the porcine pancreatic enzyme (27). Data for the basic bee venom enzyme is also provided in Fig. 1, since this enzyme has a net positive charge of +9. This enzyme shows negligible ability to hydrolyze suspensions of *M. luteus*.

Thus, overall the only human $sPLA_2$ that shows significant activity with the *M. luteus* suspension assay in addition to the group IIA enzyme is the group V enzyme, with an overall positive charge of +10. The results highlight an apparent correlation between the cationic nature of the $sPLA_2$ and the ability of the enzyme to penetrate the cell wall of *M. luteus*.

The Role of Positive Charge in Determining the Ability of Human Group IIA $sPLA_2$ to Hydrolyze the Cell Membrane of M. luteus—Suspensions of *M. luteus* provide a good model system for determining the effect of protein structure on cell wall permeability, allowing cell membrane hydrolysis. Potentially adverse effects resulting from mutagenesis of the IIA enzyme in terms of hydrolytic activity can be easily identified. Thus, prior treatment of *M. luteus* with lysozyme to disrupt the cell wall will allow unrestricted access of the enzyme to the cell membrane and the determination of full phospholipase activity.

The data described above and elsewhere (27) highlight the possible importance of the extreme cationic nature (+19) and hence surface positive charge of the IIA enzyme in allowing the enzyme to penetrate the anionic bacterial cell wall. To demonstrate the importance of this positive charge on the IIA enzyme for cell wall penetration, a number of charge reversal mutants (19, 20) were studied. Moreover, this strategy would establish whether it was the global charge or specific positively charged domains on the protein surface that were required for cell wall penetration. In this strategy of charge reversal mutagenesis, surface lysine and arginine residues were mutated to glutamate (or aspartate in the case of R-127) (19, 20), and up to five site mutations were incorporated into a single protein molecule, thus changing the net positive charge from +19 to +9.

The effect of charge reversal mutations on the hydrolysis of suspensions of *M. luteus* was determined, and the results are summarized in Fig. 3*A*. It can be clearly seen that there was a direct correlation between the loss of positive charge and the ability to hydrolyze these cells. In the case of the five-site mutations (K53E/R54E/R58E/K124E/R127D and K7E/K10E/K16E/K124E/R127D), these mutants (+9) were essentially inactive, expressing less than 1% of activity against the bacterial suspension compared with wild-type enzyme. There was no obvious requirement for specific cationic domains on the protein surface that are required for cell wall penetration, since

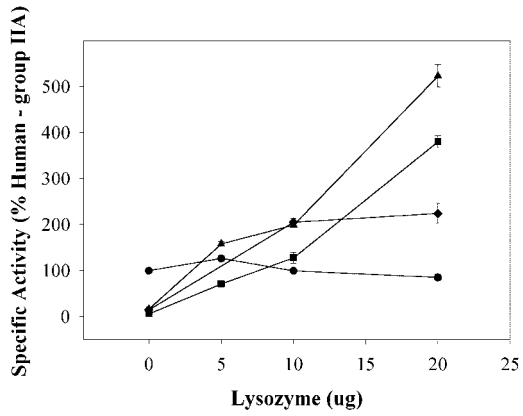


FIG. 4. **Effect of lysozyme pretreatment on the ability of native and charge reversal mutants of human group IIA $sPLA_2$ to hydrolyze cell suspensions of *M. luteus*.** Cell suspensions of *M. luteus* (1×10^7 cells/ml) were incubated with varying amounts of lysozyme (from chicken egg white) for 2 min prior to the addition of native (●) and charge reversal mutants K53E/R54E/R58E/K124E/R127D (■), K74E/K87E/R92E (▲), and R7E/K10E/K16E (♦) of human group IIA $sPLA_2$. Specific activities were determined using a fluorescence displacement assay described under "Experimental Procedures." Data shown are means ± S.D. ($n = 3$).

these mutations involved different clusters of positive charge on the protein surface (19, 20).

To confirm that such mutants were still catalytically viable, they were assayed using phospholipid vesicles prepared from DOPG as shown in Fig. 3*B*. In all cases, the expressed activity was not adversely affected compared with the native enzyme. Interestingly, several of the mutants, particularly those involving positions 7, 10, and 16, expressed enhanced activity compared with the native enzyme under these assay conditions. This phenomenon has already been seen, be it in less dramatic form, when assaying the enzyme using different phospholipid vesicles (20) and must reflect some unappreciated aspect of interfacial kinetics under these assay conditions. One possible explanation is that because the mutants will bind less tightly to anionic vesicles they are more able to "hop" between vesicles, and this allows an expression of higher enzyme activity under the assay conditions used.

To show that the charge reversal mutants are also able to hydrolyze *M. luteus* membranes in the absence of the cell wall, cells were pretreated with lysozyme prior to treatment with native or mutant enzymes. The results (Fig. 4) dramatically demonstrate the ability of charge reversal mutants to hydrolyze the cell membrane once the cell wall has been disrupted by the lysozyme treatment. Indeed, whereas the activity of the native enzyme remained high and was not significantly affected by lysozyme pretreatment, the activity of three charge reversal mutants increased dramatically and showed greater activity than the native enzyme. This phenomenon had already been seen when assaying activity with DOPG vesicles (Fig. 3*B*), but unlike with DOPG vesicles the greatest increase was seen with the K74E/K87E/R92E mutant.

The Role of Positive Charge in Determining the Ability of Human Group IIA $sPLA_2$ to Hydrolyze the Cell Membrane of S. aureus and L. innocua—It was important to confirm the principle established for *M. luteus*, that surface positive charge is critical in allowing cell wall penetration of the enzyme, in other Gram-positive bacteria. Previous studies have demonstrated that both *Staphylococcus* and *Listeria* species are sensitive to the human group IIA enzyme (10). Because only very small amounts of some charge reversal mutants were available, the study was extended using a two-site mutant (K124E/R127D) and the five-site mutant K53E/R54E/R58E/K124E/R127D. The data shown in Fig. 5*A* clearly show partial loss of ability to hydrolyze suspensions of *S. aureus* in the case of the two-site mutant and negligible activity with the five-site mutant, thus confirming the situation with *M. luteus* (Fig. 3*A*). Moreover, by using the selective cell wall protease lysostaphin to disrupt cell wall structure, it was confirmed that these charge reversal mutants were still active on the *S. aureus* cell membrane.

In the case of *L. innocua*, a similar profile was observed (Fig. 5*B*), while the overall sensitivity of this bacterium to the group IIA enzyme (10) was confirmed in terms of expressed specific activity. There is not a specific treatment available to disrupt the cell wall of *L. innocua* and to allow the expression of full enzyme activity of the mutants with the *Listeria* cell membrane.

The Anti-bacterial Properties of Human Group IB, IIA, V, and X $sPLA_2$—To confirm that the ability of the various human $sPLA_2$s to hydrolyze cell suspensions of *M. luteus* was paralleled by antibacterial activity, cultures were treated with enzyme for up to 2 h, and the bacterial viability was assessed. As can be seen in Fig. 6*A*, the IIA was clearly more effective than the group V enzyme, while under these conditions there was minimal loss of viability on treatment with the group IB and X enzymes.

The Antibacterial Properties of the Charge Reversal Mutants of Human Group IIA $sPLA_2$—The loss of hydrolyzing activity seen with the charge reversal mutants was paralleled by loss of antibacterial activity as shown in Fig. 6*B*. The two-site mutant still retained significant antibacterial activity, whereas no significant bacterial killing was seen with the five-site mutant. This same trend was seen with cultures of *S. aureus* (Fig. 6*C*) and *L. innocua* (Fig. 6*D*). However, *L. innocua* was considerably more sensitive to the IIA enzyme than *S. aureus*, while *M. luteus* sensitivity to the IIA $sPLA_2$ was intermediate between the other two bacteria. The concentrations of enzyme needed to generate Fig. 6, *B–D*, were 1.87, 20, and 0.187 μg/ml, respectively, and the 100-fold difference in sensitivity to the IIA enzyme between *Listeria* and *Staphylococcus* was entirely consistent with previous published work (10) using the $sPLA_2$ from human tears. Bactericidal assays were more sensitive in the presence of bovine serum albumin than when bovine serum albumin was omitted (data not shown), consistent with the ability of this protein to bind both fatty acids and lysophospholipids, thus reducing the rate of resynthesis of phospholipids.

DISCUSSION

The precise physiological roles of human group IIA $sPLA_2$ remain unclear, and the discovery of an increasing number of $sPLA_2$s has added to the complexity of the situation (1). The fact that all these enzymes are of similar size and can be confidently predicted to have similar crystal structures coupled to only modest differences in active site specificity focuses attention on the interfacial and other surface properties of these enzymes. Arguably, it is these major differences in surface properties that reflect the physiological roles of these groups of enzymes. Moreover, since these enzymes are secreted, it is the extracellular environment that would be expected to provide the necessary membrane surface and other factors for the enzyme to function.

In the case of the human group IIA enzyme, it is the extreme cationic nature of the protein together with an absence of an interfacial tryptophan (28) that dominates its surface properties. The enzyme binds tightly to heparin (19, 20, 29) and has a marked preference for anionic phospholipid interfaces, not normally a characteristic of the outer monolayer of the eukaryotic cell membrane. A unique property of this enzyme is its almost complete lack of ability to hydrolyze such zwitterionic plasma membranes except when using concentrations of enzyme far in

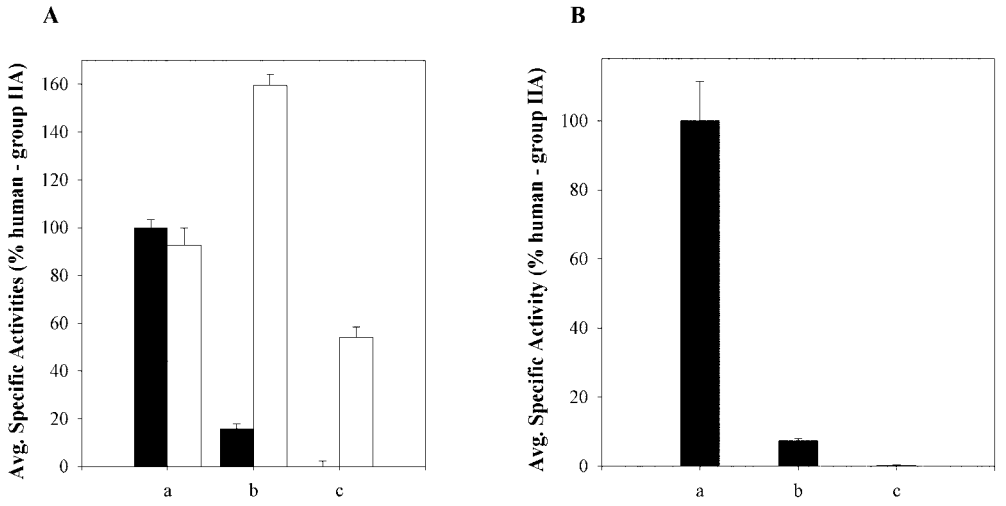


FIG. 5. **The ability of native and charge reversal mutants of human group IIA sPLA$_2$ to hydrolyze *S. aureus* (*A*) and *L. innocua* (*B*) membranes.** Specific activities were determined for native (*a*) and charge reversal mutants K124E/R127D (*b*) and K53E/R54E/R58E/K124E/R127D (*c*) of human group IIA sPLA$_2$ (150 ng) against intact (*solid bars*) and lysostaphin (430 ng)-pretreated (*open bars*) *S. aureus* (*A*) (2×10^7 cells/ml) and intact *L. innocua* (*B*) (2×10^8 cells/ml) membranes using a fluorescence displacement assay described under "Experimental Procedures." Data shown are means ± S.D. ($n = 3$).

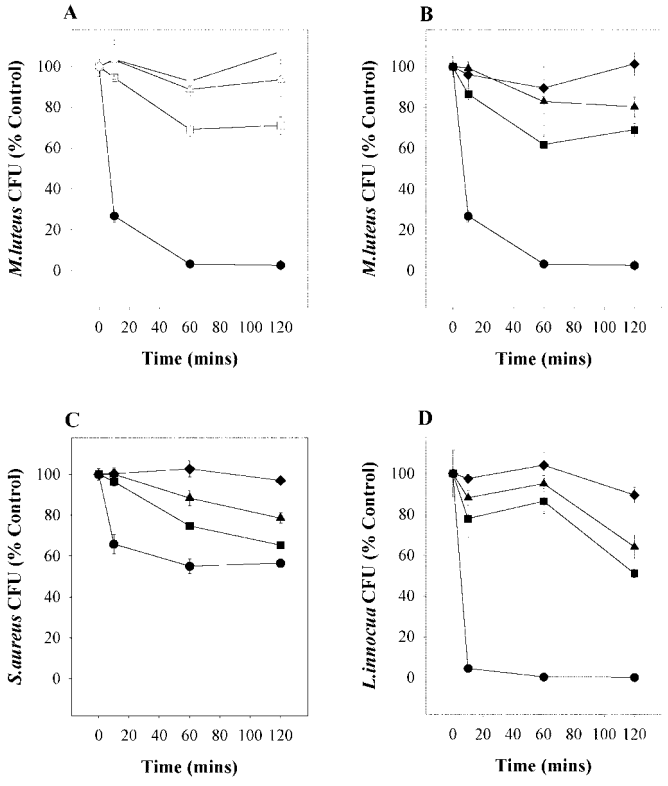


FIG. 6. ***In vitro* bactericidal activities of human sPLA$_2$s.** Bacterial viability was assessed by measuring colony-forming ability as described in "Experimental Procedures." *A*, *M. luteus* suspensions (1×10^7 cells/ml) after treatment with 1.87 μg/ml human groups IIA (●), V (□), X (△), and IB (◇) sPLA$_2$s. *B–D*, the effect of native (●) and charge reversal mutants R7E/K10E (■), K74E/K87E/R92E (▲), and K53E/R54E/R58E/K124E/R127D (◆) of human group IIA sPLA$_2$ on *M. luteus* suspensions (1×10^7 cells/ml) after treatment with 1.87 μg/ml (*B*), *S. aureus* suspensions (2×10^7 cells/ml) after treatment with 20 μg/ml (*C*), and *L. innocua* suspensions (2×10^8 cells/ml) after treatment with 187 ng/ml (*D*) of enzyme. Data shown are means ± S.D. ($n = 3$).

excess of that seen in the cell except perhaps under the most extreme inflammatory conditions (4, 14, 20, 26). Such characteristics make the enzyme particularly well designed for it to have selective antibacterial properties, because bacteria are characterized by their anionic nature in terms of cell membrane, cell wall, and, in the case of Gram-negative bacteria, the lipopolysaccharide coat.

The antibacterial potential of the group IIA enzyme has been recognized for some time (reviewed in Ref. 12), and the importance of surface positive charge in allowing penetration of the wall has been discussed (12). Other human sPLA$_2$s do not have the extreme cationic nature commensurate with this antibacterial function, although the human group V enzyme does show considerable cationic character.

In this paper, we have compared the bacterial cell membrane hydrolyzing potential of the human group IB, IIA, IIE, IIF, V, and X enzymes. The results clearly demonstrate that the group IIA has enhanced ability to hydrolyze the cell membrane of suspensions of Gram-positive bacteria, a characteristic that reflects the ability of the enzyme to penetrate the anionic peptidoglycan cell wall. Once the permeability barrier of the cell wall is removed, then all enzymes express similar activity against the bacterial cell membrane.

The bacterial cell wall is a structurally complex barrier that varies considerably from organism to organism. Therefore, the permeability properties to a large molecule such as a sPLA$_2$ will vary accordingly. In the case of *M. luteus*, this has provided a good model system, because the IIA enzyme appears to be fully permeable to the cell wall in comparison with other sPLA$_2$s, while the cell wall can be made permeable by treatment with lysozyme. Similarly, we found that *Listeria* was very sensitive to the action of the IIA enzyme. It was the most sensitive to hydrolysis of the organisms tested, confirming published data (10). In contrast, other bacteria such as *S. aureus* have a more condensed cell wall, and permeability of even the IIA enzyme is restricted and may be sensitive to the physiological state of the organism (30).

Results using suspensions of *M. luteus* have highlighted the dramatic ability of the IIA enzymes to hydrolyze the cell wall in such suspensions by making use of a real time fluorescence displacement assay that is well suited for such studies. Although an indirect assay, the fluorescence data correspond well with radioactive assays and, in particular, the measurement of phospholipid content before and after sPLA$_2$ treatment using electrospray ionization mass spectrometry (27). In contrast, the fluorescence assays highlight the greatly reduced ability of other human sPLA$_2$s to hydrolyze this organism unless the cell wall has been previously permeabilized by lysozyme treatment.

It is noteworthy that the group V enzyme, which is also a basic protein (tabulated net charge +10), has a modest ability to penetrate the cell wall. The basic bee venom enzyme (tabulated net charge +9) was included in this comparison and also was unable to demonstrate significant ability to hydrolyze *M. luteus* cell suspensions. However, the size and structure of this enzyme are sufficiently different from group I and group II enzymes to make direct comparisons more difficult.

Our understanding of the structure and function of the group IID, IIE, and X enzymes is at an early stage; however, the data presented would suggest that these enzymes do not have a bactericidal role in the body.

The major role of surface cationic charge in allowing the group IIA enzyme to penetrate the highly anionic cell wall has been confirmed by structural studies involving charge reversal mutagenesis in which surface cationic residues have been turned into anionic residues. It is clear that the loss of cell wall permeability correlated with loss of positive charge. By the time that five-site mutations had been introduced (net positive charge of 9) this permeability was less than 1% of the wild-type enzyme. This loss of permeability was monitored as reduced access to the underlying cell membrane, resulting in corresponding reduced rates of hydrolysis. Because the strategy of charge reversal mutagenesis involved different clusters of cationic residues (19), it is apparent that there are no specific cationic domains that are required to allow cell wall permeability. The direct involvement of the interfacial surface in cell wall penetration is unlikely based on the results with the five-site mutant (K53E/R54E/R58E/K124E/R127D) that involves residues distant from the interfacial binding surface (20).

The model we propose (12) involves multiple electrostatic interactions between the enzyme and the cell wall that would both increase the local concentration of enzyme at the cell surface and promote passage through anionic pores as a result of the continuous making and breaking of electrostatic bonds. Overall, the very high affinity of this enzyme for the anionic phospholipid interfaces would promote the net transfer of enzyme from the cell wall to the bacterial cell membrane.

In the case of Gram-negative bacteria such as *E. coli*, the enzyme has to first penetrate the lipopolysaccharide coat. The pioneering work of Elsbach, Weiss, and colleagues (31, 32) has already highlighted the role of bactericidal/permeability-increasing protein in this penetration, where it increases the permeability of the lipopolysaccharide coat (33, 34). This enhanced permeability would allow access of the enzyme to the cell wall, where the selective permeability of $sPLA_2$s would be expressed.

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