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Role of Charge Properties of Bacterial Envelope in Bactericidal Action of Human Group IIA Phospholipase A_2 against *Staphylococcus aureus**

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Mammalian Group IIA phospholipases A_2 (PLA_2) potently kill *Staphylococcus aureus*. Highly cationic properties of these PLA_2 are important for Ca^{2+}-independent binding and cell wall penetration, prerequisites for Ca^{2+}-dependent degradation of membrane phospholipids and bacterial killing. To further delineate charge properties of the bacterial envelope important in Group IIA PLA_2 action against *S. aureus*, we examined the effects of mutations that prevent specific modifications of cell wall (*dltA*) and cell membrane (*mprF*) polyanions. In comparison to the parent strain, isogenic *dltA*$^-$ bacteria are ~30–100× more sensitive to PLA_2, whereas *mprF*$^-$ bacteria are <3-fold more sensitive. Differences in PLA_2 sensitivity of intact bacteria reflect differences in cell wall, not cell membrane, properties since protoplasts from all three strains are equally sensitive to PLA_2. A diminished positive charge in PLA_2 reduces PLA_2 binding and antibacterial activity. In contrast, diminished cell wall negative charge by substitution of (lipo)teichoic acids with D-alanine reduces antibacterial activity of bound PLA_2, but not initial PLA_2 binding. Therefore, the potent antistaphylococcal activity of Group IIA PLA_2 depends on cationic properties of the enzyme that promote binding to the cell wall, and polyanionic properties of cell wall (lipo)teichoic acids that promote attack of membrane phospholipids by bound PLA_2.

A wide variety of mammalian peptides and polypeptides has been described that can kill staphylococci and many other Gram-positive bacteria. Of these, possibly the most potent is the 14-kDa secretory group IIA phospholipase A_2 (PLA_2)[1] (1). Levels of this enzyme sufficient for significant antibacterial action are found at a variety of sites including tears and seminal and many other inflammatory fluids, as well as within granules of certain leukocytes and platelets (2–7). The Group IIA PLA_2 can act independently and also in concert with other host defense systems (1). It has been shown that the bactericidal activity of inflammatory fluids against *Staphylococcus aureus* is due mostly to mobilized Group IIA PLA_2 (5, 8) and that killing of encapsulated *S. aureus* by whole rabbit peritoneal inflammatory exudates is also dependent on PLA_2.[2] In addition, transgenic mice with overexpressed PLA_2 show much greater resistance to *S. aureus* than their non-transgenic littermates, implying a protective role of Group IIA PLA_2 against *S. aureus* infections *in vivo* (9, 10).

The ability of Group IIA PLA_2 to attack *S. aureus* and other Gram-positive bacteria reflects the ability of the enzyme to bind to and penetrate the cell wall to gain access to phospholipids (PL) in the cell membrane. At least four steps are involved: binding of the enzyme to the bacterial cell surface, penetration of the enzyme through peptidoglycan layers, degradation of PL in the cell membrane, and activation of bacterial autolysins (11). The phospholipolytic activity of PLA_2 and, hence, ultimate bacterial killing requires calcium as a cofactor (8), but initial PLA_2 binding to the cell surface does not.[3]

Initial binding of PLA_2 to the cell surface of *S. aureus* involves electrostatic interactions[3] between PLA_2 and the bacterial cell surface. Among the more than 100 structurally related low molecular weight (~14 kDa) PLA_2 that have been characterized to date, the mammalian Group IIA PLA_2 are unique in their very high net positive charge ranging from +12 to +17. This very high net basicity is essential for the enzyme's potent bactericidal activity toward Gram-positive bacteria, principally by promoting initial interactions and penetration of the cell wall (12–14). [3] In contrast, highly cationic properties of PLA_2 are not essential for calcium-dependent catalytic activity (12) nor for the ability of structurally related 14-kDa PLA_2 to degrade PL in cell wall-depleted bacterial protoplasts (15, 16).[3] Specific bacterial binding sites of PLA_2 are not known, but probably involve cell envelope anionic moieties. Major cell envelope-associated polyanions in *S. aureus* include the cell wall lipoteichoic and teichoic acids (LTA and TA, Ref. 17) and cell membrane PL (phosphatidylglycerol, PG and cardiolipin, CL; Ref. 17). The net charge on these molecules may be regulated

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[1] The abbreviations used are: PLA_2, phospholipase A_2; BSA, bovine serum albumin; CAP, cationic antimicrobial peptides; CFU, colony-forming units; CL, cardiolipin; DAG, diacylglycerol; HBD-3, human-β-defensin-3; HBSS $^{+/-}$, Hanks' balanced salt solution: +/− Ca^{2+} and Mg^{2+}; HPLC, high pressure liquid chromatography; LTA, lipoteichoic acid; PA, phosphatidic acid; PEI, polyethylenimine; PG, phosphatidylglycerol; PL, phospholipids; TA, teichoic acid; wt, wild type.

[2] A. Foreman-Wykert, J. Lee, P. Elsbach, and J. P. Weiss, manuscript in preparation.

[3] N. S. Liang, R. S. Koduri, M. H. Gelb, and J. P. Weiss, manuscript in preparation.

by substitution of repeating glycerol (alditol)-phosphate moieties with D-alanine in LTA and TA (18, 19) or with L-lysine in PG (20). These substitutions depend on the *dlt* operon and *mprF* gene, respectively (19–21). The susceptibility of *S. aureus* to many small cationic antibacterial compounds is similarly increased in *dltA*$^-$ or *mprF*$^-$ mutants, indicating that both cell wall and cell membrane polyanions are important determinants of bacterial sensitivity to these antibacterial compounds (19, 20). Because the cationic properties of PLA_2 seem important in the interactions of the enzyme with the bacterial cell wall but not the cell membrane, we hypothesized that D-alanylation of cell wall LTA and TA would have a greater effect on bacterial susceptibility to PLA_2 than *mprF*-dependent modification of the cell membrane PG. The results presented confirm this scenario. We have made use of site-specific mutagenesis of the human Group IIA PLA_2 to further demonstrate that the higher susceptibility of the *dltA*$^-$ strain does not depend on either the net charge of the PLA_2 or higher initial binding of the enzyme to the bacterial surface of *dltA*$^-$ bacteria. Instead, the absence of D-alanine modification of LTA and TA probably results in pleitropic modifications of the staphylococcal cell wall that promote Ca^{2+}-dependent attack of bacterial PL by bound PLA_2.

EXPERIMENTAL PROCEDURES

Materials—[Ala^{8,13,18}]Magainin II amide, lysostaphin (3000 units/mg of protein), DNase I (2500 Kunitz units/mg of protein), and polyethylenimine (PEI) were purchased from Sigma Chemical Co. Human-β-defensin-3 (HBD-3) was a generous gift from Dr. Paul McCray (University of Iowa, Department of Pediatrics, Iowa City, IA). [1-^{14}C]Oleic acid was purchased from PerkinElmer Life Sciences. IODO-GEN was obtained from Pierce Chemical Co., and HP-TLC plates from Merck. PhastGels were purchased from Amersham Biosciences, GF/C glass microfiber filters from Whatman International Ltd (Maidstone, England), and bovine serum albumin (BSA) from Roche Molecular Biochemicals. RPMI 1640 was obtained from Invitrogen and Hanks balanced salt solution with (HBSS$^+$) or without (HBSS$^-$) Ca^{2+} and Mg^{2+} were obtained from Cellgro Mediatech Co. (Herndon, VA).

Bacterial Strains and Growth Conditions—Strains of *S. aureus* used were the parent SA113 (22), isogenic *mprF*$^-$, *dltA*$^-$ (AG1) mutants and their corresponding complemented strains (*mprF*$^-$ pRB *mprF* and *dltA*$^-$pRB *dltA*$^-$, respectively; Refs. 19 and 20). Bacteria were grown overnight at 37 °C in BM broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% K_2HPO_4, 0.1% glucose), washed, and resuspended in fresh medium with a starting OD_{550} of 0.05 and subcultured for 2–2.5 h until mid-log phase.

Recombinant Human PLA_2—Recombinant wild type and mutant human secretory Group IIA PLA_2, R92E, K38E/K116E (16), and D49S[3] were expressed in *Escherichia coli* and purified as described previously (12). Purity of PLA_2 was confirmed by SDS-PAGE and by reversed-phase HPLC (12). The enzymatic activity of [D49S]PLA_2 against autoclaved *E. coli* was ~0.001% of wt PLA_2.[3]

Assay of Bacterial Viability—The effect of various antimicrobial proteins and peptides on bacterial viability was determined by measuring the ability of treated bacteria to form colonies on BM agar. Typical incubation mixtures contained 10^6 or 10^7 bacteria/ml in RPMI 1640 supplemented with 10 mM Hepes (pH 7.4), 1% (w/v) BSA, and 1 mM $CaCl_2$ ± indicated protein/peptide. In assays with [Ala^{8,13,18}]Magainin II amide and HBD-3, $CaCl_2$ was omitted, since these peptides are more sensitive to inhibition by added divalent cations than is PLA_2. Incubations were carried out at 37 °C for up to 2 h. At each time point, aliquots of bacterial suspensions were serially diluted in sterile physiological saline and plated in 5 ml of molten (50 °C) BM agar. Bacterial viability was measured by counting bacterial colonies (*i.e.* colony-forming units (CFU)) after 18–24 h incubation at 37 °C.

Radiolabeling of S. aureus Lipids during Bacterial Growth—Bacterial PL were radiolableled during growth in subculture as previously described (11). Briefly, bacteria were subcultured at 37 °C to mid-logarithmic phase in BM supplemented with 1 μCi/ml of [1-^{14}C]oleic acid and 0.01% BSA, washed, resuspended in half the volume of fresh BM medium without [1-^{14}C]oleic acid, and incubated for another 20 min at 37 °C. BSA was then added to the medium to a final concentration of 0.5% (w/v), and bacteria were washed to remove remaining free oleic acid. Washed bacteria were resuspended to desired concentrations in incubation medium and used promptly.

Assay of Bacterial Phospholipid Degradation—Samples consisted of 10^7 bacteria/ml in RPMI 1640 supplemented with 10 mM Hepes (pH 7.4), 1% (w/v) BSA, and 1 mM $CaCl_2$ or in HBSS$^-$ supplemented as indicated. PL degradation products generated during PLA_2 treatment were quantitatively recovered in the extracellular medium as complexes with BSA (11). Therefore accumulation of radioactive material in extracellular supernatants after sedimentation (14,000 rpm for 4 min) of bacterial suspensions was used to routinely monitor PL degradation. To confirm that released ^{14}C-labeled material corresponded to PL degradation, lipids of *S. aureus* present in whole cell suspensions and extracellular supernatants, with and without PLA_2 incubation, were extracted using the method of Bligh and Dyer (23). Extracted lipids were dried under N_2 flow, dissolved in chloroform/methanol (2:1, v/v), and resolved by thin layer chromatography (TLC) in a chloroform/methanol/water/acetic acid (65:25:4:1, v/v/v) solvent system. Lipids were identified by comparison to migration of lipid standards and quantified by phosphorimage analysis using PhosphorImager and ImageQuant software (Amersham Biosciences, Molecular Dynamics Division). Intact bacterial PL and PL breakdown products (*i.e.* lyso-PL and free fatty acids) were quantitatively recovered in the bacterial pellet and extracellular medium, respectively. To maximize detection of lysyl-PG, extracts were collected and analyzed by TLC within 3 h of the end of the incubation. Storage of extracts for longer times before recovery of labeled lipids in the chloroform phase and TLC led to diminished recovery of lysyl-PG, but had no effect on overall recovery of intact PL and PL breakdown products. However, lyso-PG is incompletely recovered in the chloroform phase of lipid extracts because of its increased polarity.

Radioiodination of PLA_2—PLA_2 were radiolabeled with ^{125}I in tubes precoated with IODO-GEN as described previously (12, 24). Radiolabeled PLA_2 was separated from free iodide by chromatography on Sephadex G25 (Amersham Biosciences) equilibrated with 0.2 M sodium acetate buffer, pH 4.0. Recovered radiolabeled material was more than 95% trichloroacetic acid-precipitable.

Assay of PLA_2 Binding to S. aureus—Binding assays were performed with 10^7 bacteria/ml in HBSS$^-$ supplemented with 10 mM Hepes (pH 7.4) and 0.1% (w/v) BSA. To determine effects of envelope-associated Ca^{2+} and subsequent Ca^{2+}-dependent envelope damage by PLA_2 on PLA_2 binding, parallel assays were carried out ± 2 mM $MgCl_2$/2 mM EGTA. Radiolabeled 0.5 ng of ^{125}I-PLA_2 (wild type or mutant PLA_2) was mixed with 5 ng of unlabeled wild type or D49S PLA_2 (1:10) before addition to the incubation mixture to ensure exposure of bacteria to similar destructive effects of PLA_2 when Ca^{2+} was present, irrespective of the source of ^{125}I-PLA_2. Incubations were carried out in a total volume of 250 μl at 37 °C for 30 min. Bound ^{125}I-PLA_2 was collected on Whatman glass microfiber GF/C filters that had been presoaked in 0.5% PEI/20 mM Tris-HCl, pH 7.4, for 15 min. Filters were washed five times with 20 mM Tris-HCl, pH 7.4. Radioactivity associated with filters was measured in a gamma counter (Cobra II^{TM}, Packard Biosciences Co.).

Production and Assay of S. aureus Protoplasts—S. aureus was grown as described above until mid-log phase in medium containing [^{14}C]oleate. Metabolically labeled bacteria were harvested and resuspended at a final concentration of 4×10^9 bacteria/ml in Tris-buffered saline osmotically stabilized with 30% (w/v) raffinose. Lysostaphin and DNase I were added to a final concentration of 250 μg/ml and 500 μg/ml, respectively, and bacteria were incubated at a final concentration of 2×10^9 bacteria/ml, shaking slowly at 37 °C for 1 h. Protoplasts were collected by centrifugation at 4,500 rpm for 15 min at room temperature. The supernatant was removed, and protoplasts were gently resuspended in RPMI 1640 containing 1% BSA, 10 mM Hepes (pH 7.4), 1 mM $CaCl_2$, and 30% raffinose in RPMI 1640. Greater than 80% of [^{14}C]oleate-labeled bacterial PL of intact bacteria were present in the recovered protoplasts. The virtual absence of intact/viable bacteria in the recovered protoplast samples was confirmed by Gram staining and assay of CFU without raffinose.

RESULTS

Effect of dltA- and mprF-dependent Modifications of Cell Envelope Polyanions on Sensitivity of S. aureus to Human Group IIA PLA_2—It has been previously shown that *dltA*$^-$ and *mprF*$^-$ mutants of *S. aureus* are each ~10–30-fold more susceptible to killing by small cationic antimicrobial peptides (CAP) than the parent *dltA*$^+$/*mprF*$^+$ *S. aureus* strain (19, 20). When comparing effects of the Group IIA PLA_2 against wild type (wt) and the two mutant *dltA*$^-$ and *mprF*$^-$ strains, we obtained different results. Whereas *dltA*$^-$ bacteria were ~30–

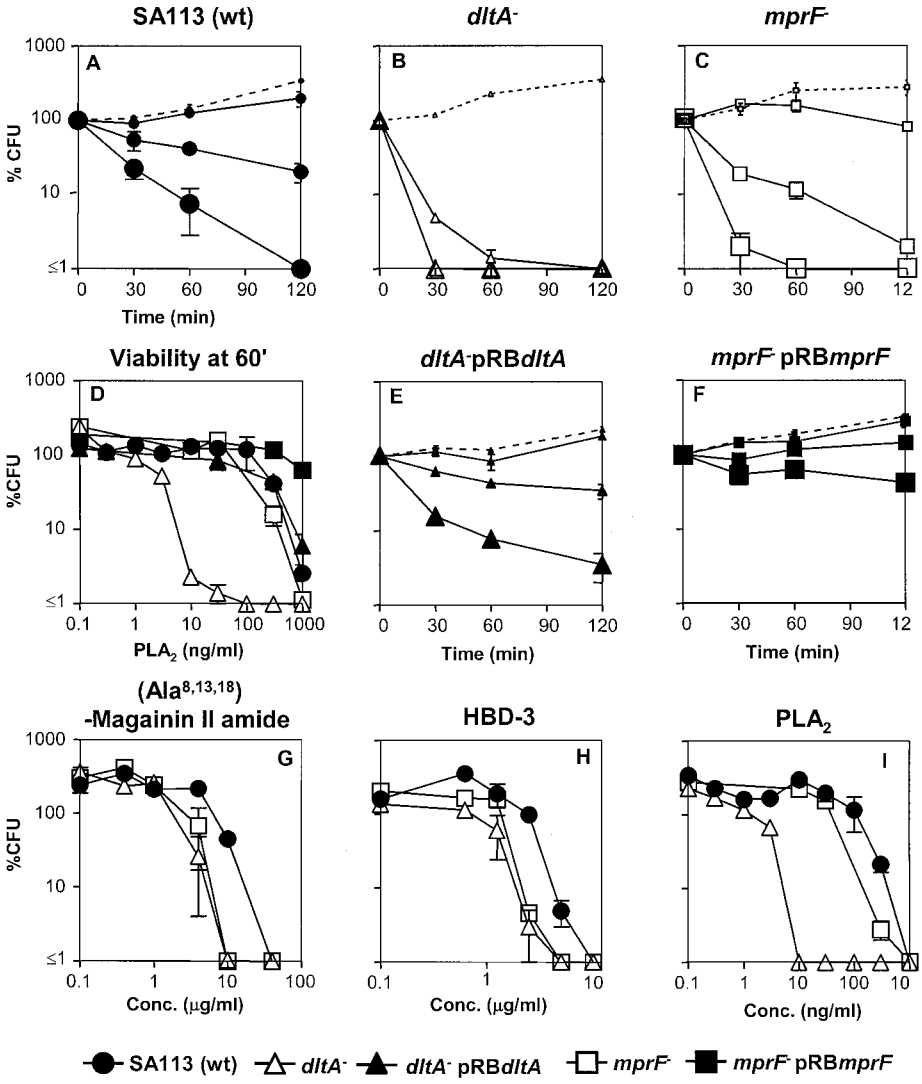

FIG. 1. **Sensitivity of *S. aureus* strains to killing by human Group IIA PLA_2, [$Ala^{8,13,18}$]Magainin II amide, and human-β-defensin-3.** Incubations contained either 10^6 bacteria/ml (*G* and *H*) or 10^7 bacteria/ml (*A–F* and *I*) with or without PLA_2 (*A–F* and *I*), [$Ala^{8,13,18}$]Magainin II amide (*G*), or human-β-defensin-3 (*HBD-3*) (*H*). All incubation mixtures contained RPMI 1640 supplemented with 10 mM Hepes (pH 7.4). PLA_2 assays were also supplemented with 1% BSA and 1 mM $CaCl_2$ whereas assays with [$Ala^{8,13,18}$]Magainin II amide and HBD-3 contained 0.1% BSA and no additional Ca^{2+}. After various times at 37 °C as indicated, bacterial viability was measured as CFU in BM agar. *A–C*, *E*, and *F* show effect of increasing concentration of PLA_2: *broken line* represents no added PLA_2; *solid lines* correspond to PLA_2-treated samples. The increasing size of symbols corresponds to increasing PLA_2 concentrations (30, 300, 1000 ng/ml). *D* shows the dose-dependent effects of PLA_2 toward the five different strains during a 60-min incubation; the different symbols correspond to those used in *A–C*, *E*, and *F*. *G–H* show results after 120 min of incubation. The results shown represent the mean of three or more experiments ± S.E.

100-fold more sensitive (Fig. 1, *A*, *B*, *D*, and *I*), *mprF*$^-$ bacteria were ≤3-fold more susceptible than wt *S. aureus* to PLA_2 killing (Fig. 1, *A*, *C*, *D*, and *I*). In contrast, under the same test conditions, both mutant strains were ~5× more sensitive to the small CAP [$Ala^{8,13,18}$]Magainin II amide and HBD-3 (Fig. 1, *G* and *H*). Higher susceptibility of the mutant *S. aureus* strains to PLA_2 was reversed by complementation with plasmid-bearing wild type *dltA* or *mprF* genes (Fig. 1, *E* and *F*). These findings demonstrate that *dltA*-dependent modifications of wall LTA and TA are much more important in resistance of *S. aureus* to PLA_2 than *mprF*-dependent modification of cell membrane PG.

Differences in Killing of wt, dltA$^-$*, and mprF*$^-$ *S. aureus by PLA_2 Parallel Differences in Phospholipid Degradation*—Killing of *S. aureus* by PLA_2 depends on rapid degradation of membrane PL due to its catalytic activity and on activation of bacterial autolysins secondary to loss of membrane PL (25). To test whether differences in killing by PLA_2 of the three *S. aureus* strains paralleled differences in PL degradation, bacterial lipids were prelabeled during growth with [^{14}C]oleic acid, and extracellular accumulation of radioactive lipid degradation products during PLA_2 treatment was measured. Dose-dependent accumulation of extracellular radioactive products during PLA_2 treatment was greatest in *dltA*$^-$ but similar in *mprF*$^-$ and wt strains, just as was observed in the killing experiments (compare Figs. 1 and 2). Release of [^{14}C]lipids in untreated samples (Fig. 2, *dashed lines*) was also greatest in the *dltA*$^-$ strain compared with the wt and *mprF*$^-$ strains, suggesting greater instability of cell envelope lipids in the *dltA*$^-$ *S. aureus* strain even in the absence of PLA_2 treatment. Increased sensitivity to PLA_2 was reversed by complementation of the mutant strains.

To verify that release of ^{14}C-labeled lipids during PLA_2 treatment corresponds to bacterial PL breakdown radiolabeled bacterial lipids in whole suspensions and extracellular supernatants of PLA_2-treated bacteria were extracted and analyzed by TLC (Fig. 3). No significant differences were observed between the profiles of labeled lipids extracted from the untreated wt and *dltA*$^-$ bacteria, whereas the labeled lipid profile of *mprF*$^-$ bacteria revealed, as expected, the absence of lysyl-phosphatidylglycerol (lysyl-PG) (Fig. 3). The predominant species in all strains was PG (Fig. 3). After treatment with 30 ng/ml PLA_2, extracts of recovered supernatants contained mainly [^{14}C]lyso-PG and to a lesser extent ^{14}C-labeled free fatty acids. At this dose, PLA_2 caused almost complete loss of intact prelabeled PL, phosphatidic acid (PA), PG, and CL in the *dltA*$^-$ strain during a 60-min incubation. In contrast, under the same conditions only a limited portion of the intact prelabeled PL species was degraded in *mprF*$^-$ and wt bacteria (Fig. 3, *whole cell suspensions*). Therefore, PLA_2-dependent PL degradation paralleled differences in sensitivity to killing by PLA_2: *dltA*$^-$ ≫ *mprF*$^-$ ≥wt. Major [^{14}C]lipids in supernatants upon PLA_2 treatment were lyso-PL, *i.e.* lyso-PG. PLA_2-independent release of [^{14}C]lipids was also greater in *dltA*$^-$ compared with *mprF*$^-$ and

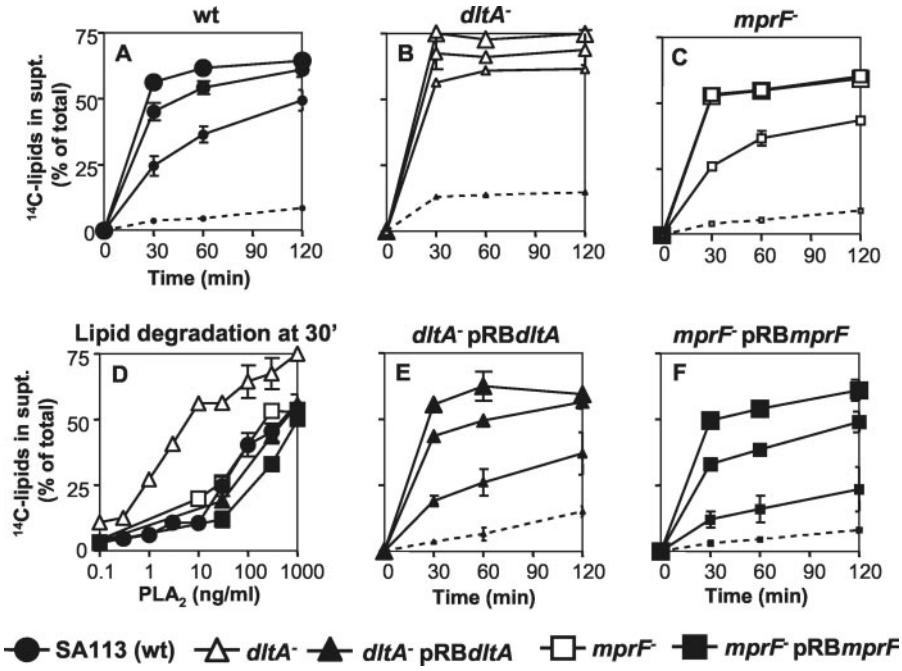

FIG. 2. **Accumulation of radioactive lipids in extracellular medium during PLA_2 treatment of *S. aureus* strains.** [^{14}C]Oleate-labeled *S. aureus* (10^7 bacteria/ml) harvested from mid-logarithmic phase were incubated in RPMI 1640 supplemented with 1 mM $CaCl_2$, 10 mM Hepes (pH 7.4), and 1% BSA in the presence or absence of PLA_2 at 37 °C. At indicated times, samples were removed to measure accumulation of ^{14}C-labeled lipids in extracellular medium as described under "Experimental Procedures." *A–C*, *E,* and *F* show effects of increasing concentrations of PLA_2 (increasing sizes of symbols represent increasing concentration of PLA_2; 30, 300, 1000 ng/ml). *D* represents the dose-dependent effect of PLA_2 toward the five different strains during 30 min of incubation where differences were the greatest. The results represent the mean of three or more experiments ± S.E.

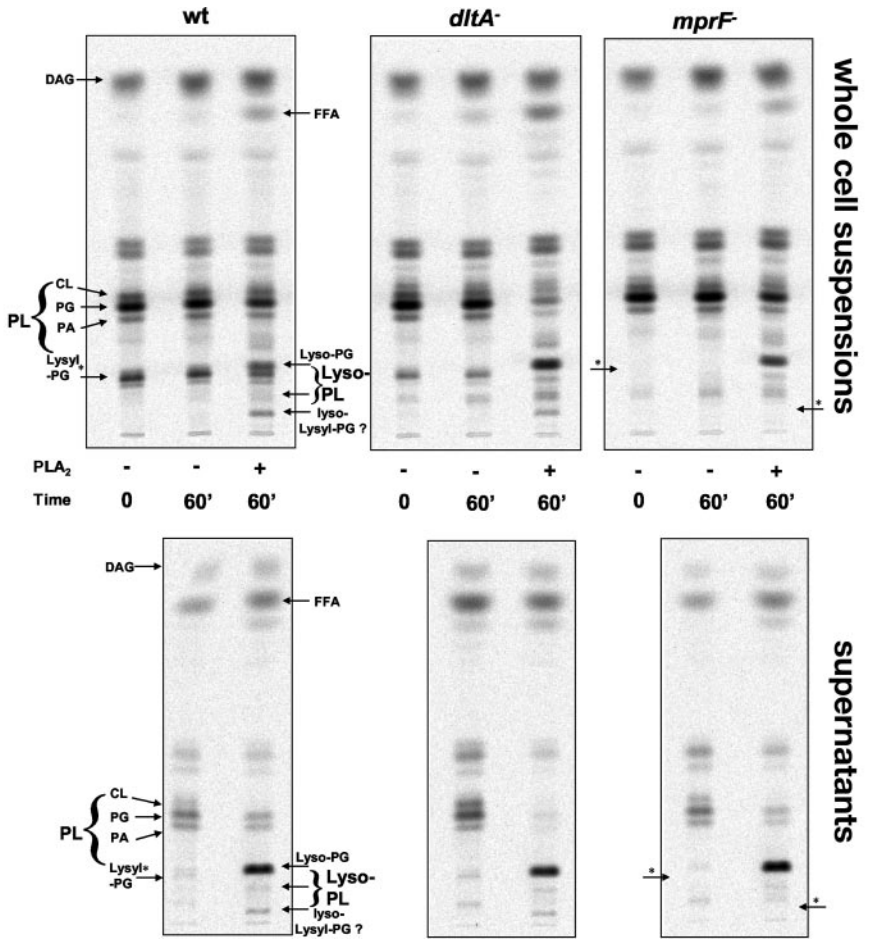

FIG. 3. **TLC analysis of total ^{14}C-labeled lipids in *S. aureus* and released [^{14}C]lipids in extracellular medium during PLA_2 treatment of bacteria.** Whole cell suspensions or extracellular supernatants recovered from [^{14}C]oleate-labeled *S. aureus* (10^7 bacteria/ml) in RPMI 1640 supplemented with 10 mM Hepes (pH 7.4), 1 mM $CaCl_2$, and 1% BSA ± 30 ng/ml PLA_2 were extracted and analyzed by TLC as described under "Experimental Procedures." Major labeled lipids present before (time, 0 min) and after (time, 60 min) incubation ± PLA_2 are indicated as determined by standards. Approximately the same amount of [^{14}C]lipids (cpm) were applied per lane. Whole cell suspensions were extracted the same day to maximize recovery of lysyl-PG.

wt bacteria, but [^{14}C]lipids in these supernatants were mainly intact lipids and free fatty acids (Fig. 3).

Cell Wall-depleted Membrane Protoplasts from wt, dltA$^-$, and mprF$^-$ S. aureus Are Equally Sensitive to PLA_2—Differences in PL degradation by PLA_2 of intact wt, *dltA*$^-$, and *mprF*$^-$ *S. aureus* could reflect either greater access of the enzyme to the membrane of more sensitive (*i.e. dltA*$^-$ ≫*mprF*$^-$, wt) bacteria or greater sensitivity of PL within the membrane of these more sensitive bacteria. To distinguish between these two possibilities we prepared bacterial protoplasts from wt, *dltA*$^-$, and *mprF*$^-$ bacteria and tested their susceptibility to PLA_2 hydrolytic activity as described under "Experimental Procedures." Recovery of bacterial membranes in the form of protoplasts was ~80%, as deduced from recovery of ^{14}C-labeled material. Con-

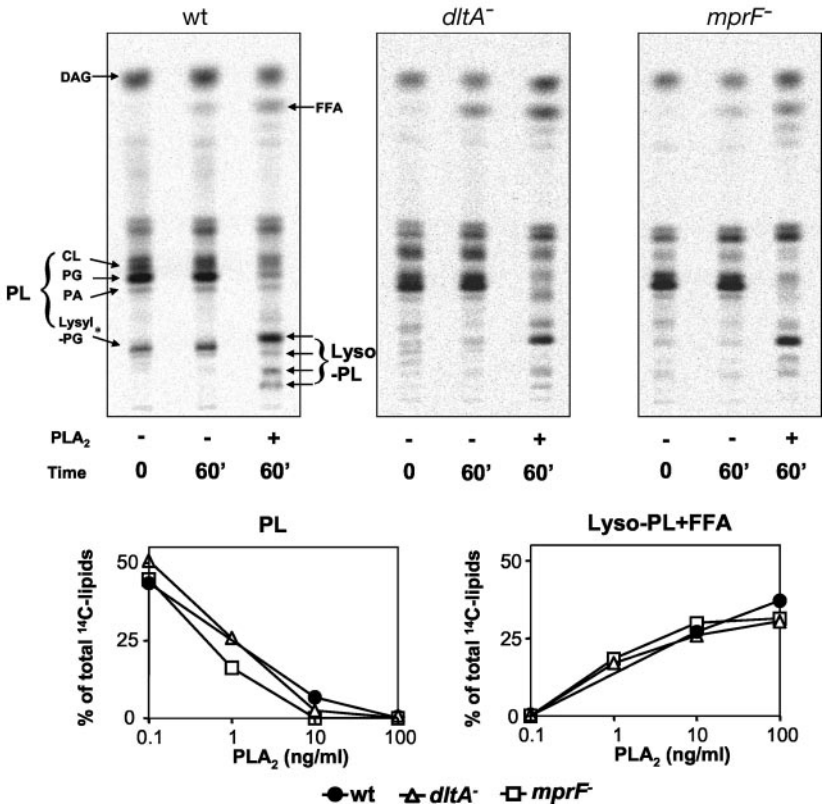

FIG. 4. **Sensitivity of cell wall-depleted membrane protoplasts from wt, dltA⁻, and mprF⁻ *S. aureus* to PLA_2 degradation.** Membrane protoplasts derived from 10^7 bacteria were incubated with increasing concentrations of PLA_2 for 60 min at 37 °C in RPMI 1640, supplemented with 10 mM Hepes (pH 7.4), 1% BSA, 1 mM $CaCl_2$, and osmotically stabilized with 30% raffinose. Lipids before (0′) and after (60′) incubation in the presence or absence of PLA_2 were analyzed by TLC (*upper panels*). Densitometry analyses are shown in the *lower panels*. The amount of PLA_2 used for the TLC analyses that are shown was 10 ng/ml. Note that appearance of lyso-PL+FFA is less than disappearance of PL from protoplasts because of incomplete recovery of lyso-PG in the chloroform phase of lipid extracts (see "Experimental Procedures").

tamination with intact bacteria, as assessed by plating protoplasts on BM agar without 30% raffinose, was ≤0.001%. The radiolabeled lipid pattern of protoplasts prior to PLA_2 treatment resembled that of intact bacteria (compare Figs. 3 and 4, *first lanes* in each set). In contrast to intact bacteria, protoplasts of all three strains were equally sensitive to PLA_2-dependent PL degradation (Fig. 4). This indicates that neither *dltA*-dependent modification of cell wall TA and LTA nor *mprF*-modification of cell membrane PG affects the sensitivity of membrane PL to PLA_2, when the cell wall is removed. Comparison of the degradative activity of PLA_2 toward intact bacteria and protoplasts (Fig. 5) shows that the resistance conferred by the cell wall in wt and *mprF*⁻ bacteria is absent in the *dltA*⁻ strain.

Increased Sensitivity of dltA⁻ Bacteria Does Not Depend on Net Charge of PLA_2—Previous studies indicated that *dltA*⁻ *S. aureus* are more sensitive to a wide range of cationic antibacterial compounds (19). To test if differences in sensitivity of wild type and *dltA*⁻ *S. aureus* are dependent on the net positive charge of the antibacterial compound, we compared the bactericidal potency of the wild type PLA_2 and two mutant PLA_2, R92E and K38E/K116E (16), against both *S. aureus* strains. As previously shown in other *dltA*⁺ strains of *S. aureus*[3] (15), the bactericidal potency of R92E and K38E/K116E PLA_2 was much less (respectively, 10-fold and 100-fold reduced) than that of the wild type PLA_2 (Fig. 6, *left panel*). Nevertheless, the *dltA*⁻ mutant strain was ~30 times more susceptible to killing by each of the PLA_2. These results demonstrate that the absence of *dltA*-dependent cell wall modifications renders *S. aureus* more susceptible to killing, not only by wt PLA_2, but also by mutant enzymes with reduced net positive surface charge. Hence, within this range of net charge of the PLA_2 (+15 → +11; (Lys+Arg) − (Glu+Asp)), effects of *dltA*-dependent modifications of the bacterial cell wall on bacterial sensitivity to PLA_2 are independent of the net charge of the PLA_2.

Effect of PLA_2 Net Charge and dlt-dependent Modifications on PLA_2 Binding to the Bacterial Surface—Because the absence of D-alanylation of LTA and TA is believed to increase the net negative charge of the bacterial surface (18, 19), the increased sensitivity of the *dltA*⁻ *S. aureus* to PLA_2 could simply reflect greater binding of PLA_2 to the *dltA*⁻ mutant strain. We therefore compared binding of ^{125}I-PLA_2 to the two strains. Initial PLA_2 binding was measured in Hepes-buffered $HBSS^-$ supplemented with 2 mM $MgCl_2$/2 mM EGTA (Mg^{2+}·EGTA) to chelate ambient and cell envelope-associated Ca^{2+} and thereby preclude Ca^{2+}-dependent envelope disruption by added PLA_2 (Fig. 7*A*). Under these conditions, PLA_2 binding to *S. aureus* declined with reduced net charge of the PLA_2 (wt > R92E > K38E/K116E), but there was no significant difference in binding of wt or mutant PLA_2 to the parent and *dltA*⁻ strains. Similar results were obtained using 10^7 or 10^8 bacteria (not shown) except that PLA_2 binding was higher when more bacteria were present. In contrast, when envelope-associated Ca^{2+} was retained by carrying out incubations in $HBSS^-$ without EGTA, PLA_2 binding was much greater to the *dltA*⁻ bacteria (Fig. 7*B*). This increase in PLA_2 binding when Ca^{2+} is present was seen with wt but not with a catalytically inactive mutant D49S PLA_2 (Fig. 7*C*) indicating that increased PLA_2 binding is secondary to Ca^{2+}-dependent envelope damage (which is greater in the *dltA*⁻ strain; Fig. 2). These results demonstrate that Ca^{2+}-independent initial binding depends on cationic properties of the PLA_2 (wt ≥ R92E > K38E/K116E) but is not significantly different than the wild type and *dltA*⁻ *S. aureus* strains.

Bound PLA_2 Is More Active against dltA⁻ S. aureus—The fact that initial PLA_2 binding is not different from wt and *dltA*⁻ *S. aureus* implies that the activity of bound PLA_2 must be greater toward the *dltA*⁻ bacteria. To test this hypothesis more directly, we took advantage of the findings described above, namely that in the presence of Mg^{2+}·EGTA, similar amounts of enzyme bound to the two strains. After an initial 30-min incubation of [^{14}C]oleate-prelabeled bacteria with PLA_2 in the presence of Mg^{2+}·EGTA, the bacteria were washed and resus-

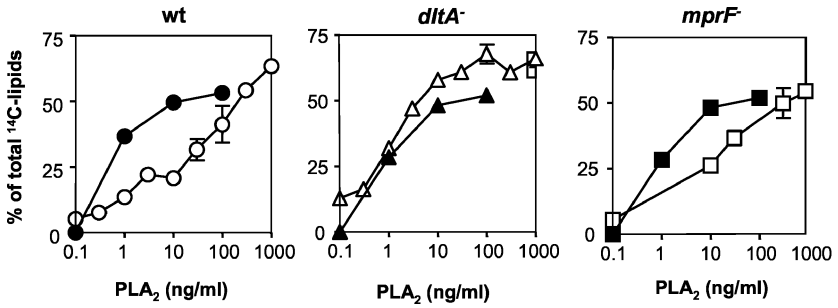

FIG. 5. **Comparison of PLA_2 activity toward protoplasts versus intact *S. aureus* from wt, dltA$^-$ and mprF$^-$ strains.** Effects of increasing concentrations of PLA_2 toward [^{14}C]oleate-labeled bacteria (10^7/ml) and protoplasts (10^7 equivalents/ml) where measured as described in the legends to Figs. 2 and 4. Incubations were for 60 min at 37 °C. PL degradation in the protoplasts was monitored by disappearance of PL observed by TLC whereas PL degradation of bacteria was measured either as accumulation of ^{14}C-labeled lipids in extracellular medium or disappearance of PL observed by TLC. All data are expressed as percent of total ^{14}C-labeled lipids. *Open symbols* represent PL degradation in intact bacteria and *closed symbols* represent degradation of PL in protoplasts at different PLA_2 concentrations.

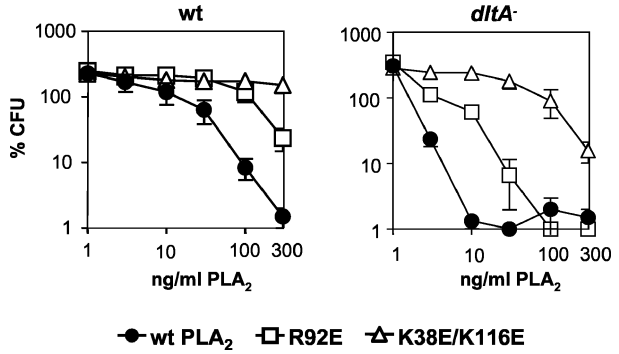

FIG. 6. **Sensitivity of wt and dltA$^-$ bacteria to wt, R92E, and K38E/K116E PLA_2.** *S. aureus* (10^6/ml) in RPMI 1640, supplemented with 10 mM Hepes (pH 7.4), 1% BSA, and 1 mM $CaCl_2$ were incubated with various doses of either wt PLA_2, R92E, or K38E/K116E. After 120 min of incubation at 37 °C, bacterial viability was measured as CFU. The CFU of experimental samples is expressed as percent of the initial bacterial inoculum. The results shown represent the mean ± S.E. of three or more independent experiments.

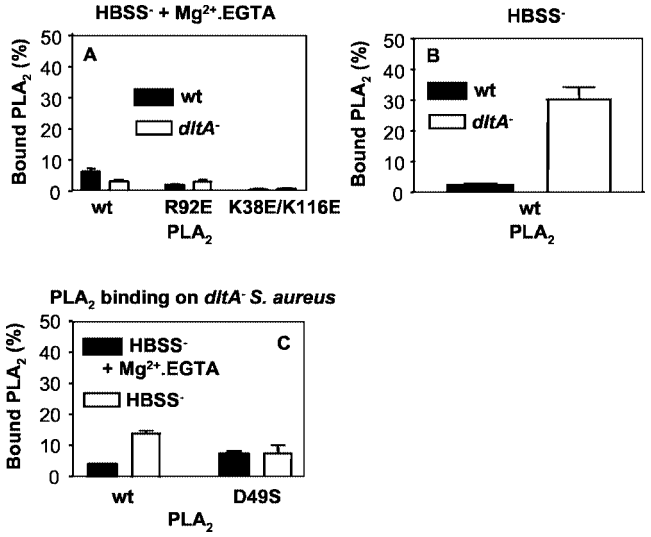

FIG. 7. **Effect of PLA_2 net charge and dltA-dependent modifications on PLA_2 binding to the bacterial surface in the presence or absence of Ca^{2+}.** 10^7 bacteria/ml (wt, *dltA*$^-$) were incubated in HBSS$^-$ supplemented with 10 mM Hepes (pH 7.4), 0.1% BSA, with (*A* and *C*) and without (*B* and *C*) 2 mM $MgCl_2$ and 2 mM EGTA (Mg^{2+}·EGTA). ^{125}I-labeled PLA_2 (wt and mutants) and unlabeled PLA_2 (wt, *A* and *B*; D49S, *C*) were added (1:10) as described under "Experimental Procedures," and incubation was carried out at 37 °C for 30 min. Bound ^{125}I-labeled PLA_2 was collected by membrane filtration and measured in a gamma-counter. The results are presented as percent of added ^{125}I-PLA_2 bound to the bacteria and represent the mean of 2–8 experiments ± S.E. Note that in *B*, only binding of wt PLA_2 to wt and *dltA*$^-$ *S. aureus* is shown.

pended in HBSS$^+$ (*i.e.* HBSS$^-$ supplemented with Ca^{2+} and Mg^{2+}). As expected, in the presence of Mg^{2+}·EGTA, PLA_2 was inactive, and there was little or no PLA_2-dependent accumulation of extracellular radioactive degradation products from either strain (compare *left bars* of Fig. 8, *A* and *B*). However, after washing the bacteria to remove unbound PLA_2 and subsequent resuspension and incubation in HBSS$^+$ (containing Mg^{2+}/Ca^{2+}), PL degradation and killing (data not shown) was much greater in the mutant strain than in the parent *S. aureus* demonstrating much greater Ca^{2+}-dependent activity of prebound PLA_2 toward *dltA*$^-$ *S. aureus* (Fig. 8*A*, *center*). Under these experimental conditions, effects of prebound PLA_2 following Ca^{2+} addition nearly matched the activity of the enzyme against the two strains when incubations were carried out in medium containing Ca^{2+} throughout (Fig. 8*A*, *right bars*). In conclusion, bound PLA_2 displays greater Ca^{2+}-dependent activity toward *dltA*$^-$ *S. aureus*. This difference largely accounts for the greater sensitivity of *dltA*$^-$ bacteria for this cationic antibacterial enzyme.

DISCUSSION

In the present study, we have shown, that in contrast to the wt and *mprF*$^-$ strains, *dltA*$^-$ *S. aureus* is 30–100-fold more sensitive to killing by human Group IIA PLA_2. Higher susceptibility of *dltA*$^-$ *S. aureus* is independent of the net charge of the PLA_2 and is not due to greater initial binding of the enzyme to the bacterial surface, but rather is due to greater Ca^{2+}-dependent activity of bound PLA_2.

It has been previously shown (19, 20) that *dltA*$^-$ and *mprF*$^-$ *S. aureus* mutants are each 10–30× more susceptible to CAP such as [Ala^{8,13,18}]Magainin II amide and human defensins, suggesting the importance of both cell wall modifications of LTA/TA and cell membrane modifications of PG in bacterial resistance to these cationic compounds. We have reproduced these findings although under our experimental conditions the *dltA*$^-$ and *mprF*$^-$ mutants are each only ~5× more sensitive than wt *S. aureus* to [Ala^{8,13,18}]Magainin II amide and HBD-3 (Fig. 1, *G* and *H*). In contrast, disruption of the *dltA* locus increases bacterial sensitivity to Group IIA PLA_2 nearly 100-fold whereas disruption of the *mprF* locus has much less effect (only ~3×; Fig. 1, *D* and *I*). Overall, these findings indicate that *dltA*-dependent modification of bacterial cell wall LTA and TA has a much greater effect on sensitivity of *S. aureus* to the antibacterial action of PLA_2 than do *mprF*-dependent modifications of the cell membrane PG. The contrasting effects of *dltA*- and *mprF*-dependent modifications of cell envelope polyanions on the action of PLA_2 and that of non-catalytic cationic antibacterial peptides suggest differences in the determinants of action of these antibacterial compounds. Such differences

may have important biologic as well as mechanistic implications especially at anatomic sites (*e.g.* mucosal secretions, inflammatory exudates; 1, 2, 8, 27) where non-catalytic peptides and PLA_2 are jointly present and could act in synergy (5).[4]

The properties of Group IIA PLA_2 important in antibacterial action differ in many ways from those of CAP. The PLA_2 has enzymatic activity that is essential for its antibacterial activity and probably explains its much greater antibacterial potency against *S. aureus* and other Gram-positive bacteria as compared with CAP (Fig. 1, *G–I* and Refs. 5 and 8). In contrast to many CAP, the PLA_2 is fully active in incubation media that contain physiological extracellular salt concentrations, including divalent cations, (8, 26, 27). The reduced effects of *dltA*$^-$ and *mprF*$^-$ mutations on [Ala^{8,13,18}]Magainin II amide and HBD-3 activity in our experiments might in fact be due to the higher salt concentrations present in our incubation media. Higher salt concentration could decrease activity of salt-sensitive cationic peptides and function as counter ions to shield polyanions of the bacterial cell envelope, thereby reducing initial binding of cationic peptides to the bacterial cell wall.

The mechanism of antibacterial action of CAP and PLA_2 is also different. For both, the initial step is interaction between cationic groups in the peptide/polypeptide and anionic moieties in the bacterial cell wall, followed by penetration through the cell wall to reach the bacterial membrane. Subsequent interactions at the bacterial membrane, however, likely differ. Killing by CAP is generally believed to require membrane insertion of the peptide (28–31) whereas PL degradation and killing by PLA_2 can be mediated by protein bound to the extracytoplasmic surface of the membrane (32–34). Membrane insertion of CAP may require very extensive electrostatic interactions between the peptide and negatively charged membrane lipids to trigger an amphiphilic transition of the peptide and membrane insertion (for review see Ref. 35). In contrast, PLA_2 membrane interactions may be satisfied by more limited electrostatic interactions and not be affected by even marked variations in either enzyme or substrate/membrane interface net charge. The closely similar activity of natural and mutant PLA_2 charge variants against cell wall-depleted membranes of Gram-positive bacteria (Fig. 4 and Ref. 15) and the similar sensitivity to PLA_2 of membrane protoplasts derived from wt, *dltA*$^-$, and *mprF*$^-$ *S. aureus* (Fig. 4) support this view. Once bound at the membrane interface, closely related PLA_2 also show limited substrate (head group) specificity (36). We observed no difference in degradation of PG and lysyl-PG by PLA_2 in wt and *dltA*$^-$ bacteria/protoplasts (Figs. 3 and 4 and Ref. 25), and differences in membrane PG content between wt and *mprF*$^-$ strains may be too limited to have more global effects on PLA_2 action. As the sensitivity of membrane protoplasts to PLA_2 is the same, whether or not lysyl-PG is present, even the modest effect of *mprF* disruption on bacterial PLA_2 sensitivity may not be due to changes in membrane properties but other, as yet unidentified, *mprF*-dependent bacterial alterations. The slightly greater resistance conferred by overexpression of *mprF*

[4] K. Zarember, P. Elsbach, and J. P. Weiss, manuscript in preparation.

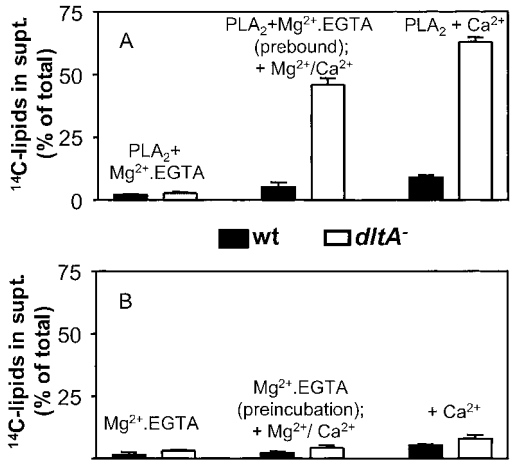

FIG. 8. **Bound PLA_2 is more active against dltA *S. aureus*.** *S. aureus* strains (10^7/ml) prelabeled with [^{14}C]oleate were incubated at 37 °C with (*A*) or without (*B*) PLA_2 (10 ng/ml), for 120 min in HBSS$^-$ supplemented with 2 mM $MgCl_2$ and 2 mM EGTA (Mg^{2+}·EGTA) (*left bars*), in HBSS$^+$ (*right bars*) or first for 30 min in HBSS$^-$ supplemented with Mg^{2+}·EGTA, washed to remove unbound PLA_2 and incubated an additional 90 min in HBSS$^+$ (*center bars*). All incubation mixtures also contained 10 mM Hepes (pH 7.4) and 0.1% BSA. At the end of the total incubation time (120 min), accumulated labeled lipids in the supernatant were measured as described before and expressed as percent of total labeled lipids present initially. The results shown represent the mean of three or more experiments ± S.E.

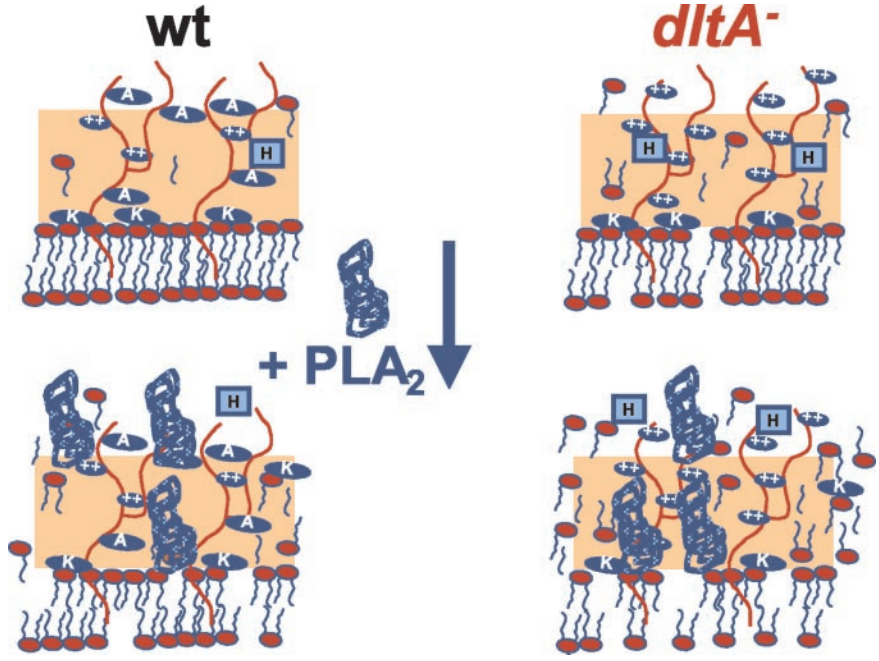

FIG. 9. **Possible effects of D-alanylation of LTA and TA on sensitivity of *S. aureus* to Group IIA PLA_2.** Alanylation (*A*) of LTA and TA may be important in maintaining barrier properties of the cell wall to macromolecules such as PLA_2. The absence of alanylation may allow easier penetration of the PLA_2 to the cell membrane either by steric effects and/or increased electrostatic binding of autolysins (*H*, hydrolases). Highly cationic PLA_2 may displace cell wall-associated autolysins leading to localized cell wall degradation and easier penetration of the PLA_2. Absence of alanylation of LTA and TA in *dltA*$^-$ bacteria may also provide a denser network of negative charges facilitating movement of bound PLA_2 and/or increased binding of divalent cations (++), which could be displaced upon binding of PLA_2 and be locally available for Ca^{2+}-dependent antibacterial activity of bound PLA_2. *Blue*, basic; *red*, acidic; *K*, Lysine.

(Fig. 1*F*) is not associated with changes in membrane content of lysyl-PG. Lysyl-PG represents 16.7 and 17.2% of total ^{14}C-membrane PL in *mprF*$^+$ and plasmid-overexpressing strains, respectively (data not shown), implying that effects on PLA_2 (CAP?) sensitivity could be caused by other *mprF*-dependent bacterial alterations.

Previous studies have indicated that increased activity of CAP toward *dltA*$^-$ *S. aureus* reflect increased binding of the cationic peptides to the cell wall LTA and TA not masked by covalently substituted D-alanine (19). In contrast, our findings indicate that increased PLA_2 activity toward these bacteria is not caused by increased binding but rather by increased Ca^{2+}-dependent activity of the bound PLA_2. Whereas binding of PLA_2 was nearly the same to wt and *dltA*$^-$ bacteria under calcium-free conditions (Fig. 7*A*), the antibacterial activity of the bound PLA_2, subsequently triggered by addition of Ca^{2+}, was much greater in the mutant strain (Fig. 8*A*) demonstrating directly the greater activity of PLA_2 bound to *dltA*$^-$ bacteria. PLA_2 binding to the mutant strain was much greater when binding was measured in the presence of the envelope associated Ca^{2+} (Fig. 7, *B* and *C*) but without appreciably increasing antibacterial activity (Fig. 8*A*) indicating that, under these conditions, increased binding did not significantly affect PLA_2 activity. In model membranes such as PL vesicles, the generation *in situ* of PL breakdown products can increase the avidity of PLA_2-membrane interactions (37). Therefore, the increased binding of PLA_2 to the mutant strain we observed when calcium was present, might simply reflect differences induced, directly or indirectly, by greater membrane damage within the envelope of the *dltA*$^-$ bacteria. In support of this view are findings presented in Fig. 7*C* showing that increased (Ca^{2+}-dependent) binding is only observed with wt and not with a catalytically inactive mutant (D49S) of PLA_2 despite similar Ca^{2+}-independent binding of wt and D49S PLA_2. Thus, increased binding of PLA_2 to the mutant strain when Ca^{2+} is present is much more likely a secondary consequence of increased activity and not itself a determinant of greater activity.

We were able to distinguish the effects of changes of bacterial envelope structure on initial PLA_2 binding and activity of bound enzyme, because these events can be completely separated by experimental manipulation of the presence or absence of Ca^{2+} (this study).[3] It is not known if similar separation of initial CAP binding and cytotoxicity can be experimentally achieved. Therefore it is not yet possible to discern whether increased CAP binding to *dltA*$^-$ *S. aureus* previously reported might also be secondary to greater susceptibility of the mutant bacteria to CAP-induced envelope damage or truly reflect higher initial CAP binding.

Effects of disruption of *dltA*-dependent LTA and TA modifications on initial binding of CAP or PLA_2 are also likely to depend on the presence of electrolytes in the incubation medium, especially Mg^{2+} and Ca^{2+}, which are known to bind with relatively high affinity to unsubstituted repeating glycerol phosphate moieties (38, 39). For this reason, we supplemented the incubation medium with Mg^{2+} when Ca^{2+} was depleted by EGTA. In contrast, earlier studies used divalent cation-poor media creating conditions more favorable for CAP binding to unsubstituted LTA and TA in the *dltA*$^-$ bacteria.

We do not, as yet, understand how *dltA*-dependent modifications affect activity of bound PLA_2. It has been shown by Peschel's group that *dltA*-dependent modifications neither affect overall LTA and TA content, the amount of *N*-acetyl glucosamine in TA nor overall surface protein content (19, 40). However, it is possible that less substitution of the polyanionic LTA and TA with D-alanine, while unimportant for initial PLA_2 attachment to the cell surface, permits greater penetration of the bound enzyme to the cell membrane. LTA and TA form a negatively charged network that connects the bacterial membrane and cell wall (17). Depending on concentrations of ambient counter ions, this network could be more densely anionic in the *dltA*$^-$ strain and could facilitate penetration of PLA_2 through anionic pores of the cell wall by "electrostatic steering" (41). In the absence of surrounding cell wall layers, such steering may be unnecessary rendering membrane protoplasts from wt and *dltA*$^-$ bacteria equally sensitive to added PLA_2 despite differences in remaining membrane-associated LTA. The similar effect of *dltA*$^-$ disruption on each of the PLA_2 charge variants studied (Fig. 5), suggests that effects not directly related to electrostatic interactions between PLA_2 and LTA/TA may be more important. These could include changes in packing of LTA and TA chains, binding of cationic autolysins and/or levels of cell wall-bound Ca^{2+} (see Fig. 9).

We believe that our studies add significant new insight concerning the mechanism of PLA_2 antibacterial action and of cationic peptides, more generally. It should be noted that the seminal studies of Beers *et al.* (15) have previously shown the importance of PLA_2 net positive charge in permitting enzyme penetration of the cell wall for attack of bacterial membrane PL of intact bacteria. However, this study did not reveal more precisely how enzyme positive charge facilitated access of PLA_2 to the bacterial cytoplasmic membrane. Our studies show a correlation of enzyme positive charge and antibacterial potency with Ca^{2+}-independent binding and describe an experimental approach that permits unambiguous assessment of initial enzyme binding to the bacterial cell wall by precluding subsequent Ca^{2+}-dependent envelope alterations. The use of this method has also permitted us to recognize that the effect of modification of cell wall polyanions on PLA_2 antibacterial activity is, unexpectedly, not on PLA_2 binding but rather on the activity of PLA_2 once bound to the bacterial envelope. The contrasting effects of mutational alterations of PLA_2 and bacterial surface charge properties has revealed the likely importance of both initial enzyme-cell wall electrostatic interactions, regulated by enzyme charge properties, and subsequent movement and actions of bound enzyme, regulated by cell wall charge properties, in the antibacterial action of PLA_2.

Whatever the mechanism of greater PLA_2 activity against *dltA*$^-$ *S. aureus*, it is apparent that this modification could play a substantial role in regulation of bacterial sensitivity to this potent host defense protein (42). Effects of *dltA*-dependent cell wall modifications are not limited to modulating bacterial sensitivity to antimicrobial peptides and polypeptides. Biofilm formation, adhesion, acid sensitivity, protein folding, and autolytic activity are also affected (42–48), implying pleitropic effects of this single cell wall modification on cell envelope structure and function. Of particular interest is recent evidence of an apparent novel two-component system involved in regulation of the *dlt* operon (49, 50). This finding strongly suggests that alanylation may be a regulated modification of the cell wall LTA and TA. If so, it is tempting to speculate that environmental cations, including host defense cationic peptides and polypeptides, could serve as environmental cues to regulate these cell wall modifications and thereby enhance bacterial resistance and persistence in potentially adverse environments such as the host.

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