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Bactericidal Properties of Human and Murine Groups I, II, V, X, and XII Secreted Phospholipases A_2*

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Group IIA secreted phospholipase A_2 (sPLA2) is known to display potent Gram-positive bactericidal activity *in vitro* and *in vivo*. We have analyzed the bactericidal activity of the full set of recombinant murine and human groups I, II, V, X, and XII sPLA2s on *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli*. The rank order potency among human sPLA2s against Gram-positive bacteria is group IIA > X > V > XII > IIE > IB, IIF (for murine sPLA2s: IIA > IID > V > IIE > IIC, X > IB, IIF), and only human group XII displays detectable bactericidal activity against the Gram-negative bacterium *E. coli*. These studies show that highly basic sPLA2s display potent bactericidal activity with the exception of the ability of the acidic human group X sPLA2 to kill Gram-positive bacteria. By studying the *Bacillus subtilis* and *S. aureus* bactericidal potencies of a large panel of human group IIA mutants in which basic residues were mutated to acidic residues, it was found that: 1) the overall positive charge of the sPLA2 is the dominant factor in dictating bactericidal potency; 2) basic residues on the putative membrane binding surface of the sPLA2 are modestly more important for bactericidal activity than are other basic residues; 3) relative bactericidal potency tracks well with the ability of these mutants to degrade phospholipids in the bacterial membrane; and 4) exposure of the bacterial membrane of Gram-positive bacteria by disruption of the cell wall dramatically reduces the negative effect of charge reversal mutagenesis on bactericidal potency.

The secreted phospholipases A_2 (sPLA2s)[1] comprise a large family of water-soluble enzymes that have the ability to bind to membrane surfaces as a prelude to the hydrolysis of the *sn*-2 ester of membrane phospholipids (1). Pancreatic sPLA2 (group IB sPLA2) was the first of these enzymes to be purified and extensively studied as a paradigm for interfacial enzymology. This enzyme is synthesized by pancreatic acinar cells and functions as a digestive enzyme within the small intestine. Group IIA sPLA2 is regarded as an acute phase protein (2) whose concentration increases in blood plasma in inflammatory diseases, including infections (3). This enzyme is capable of liberating arachidonic acid from membrane phospholipids for the biosynthesis of the eicosanoids in mammalian cells including fibroblastic and endothelial cells (4, 5). The physiological function of group IIC sPLA2 in the mouse (group IIC is present as a pseudogene in humans) (6) is not known. Group V sPLA2 plays a role in arachidonate release for eicosanoid biosynthesis in mast and macrophage cell lines (7, 8). Recently, several new murine and human sPLA2s have been cloned and shown to have lipolytic activity (groups IID, IIE, IIF, III, X, and XII) (9–11). The functions of these enzymes remain to be determined, but the fact that they have distinct tissue distribution profiles supports the proposal that they have distinct functions.

Human group IIA sPLA2 kills Gram-positive bacteria, such as staphylococci and streptococci (12, 13), *in vitro*. Group IIA sPLA2 purified from the mouse intestine is also able to kill both *E. coli* and *Salmonella* subspecies (14). Group IIA sPLA2 was recently identified as the principal Gram-positive bactericidal agent in human tears and is able to kill several *S. aureus* subspecies and *Listeria monocytogenes* but not *Enterobacter faecium, Bacillus subtilis*, or *Streptococcus pneumoniae* (15).

Enzymatic activity (phospholipolysis) is required for the bactericidal activity of mammalian group IIA sPLA2 against Gram-positive (12, 14) and Gram-negative (16) bacteria. It has been suggested that bacterial envelope sites engaged in cell growth may represent preferential sites for the action of group IIA sPLA2 against Gram-positive bacteria (17). Phospholipid hydrolysis and bacterial killing were more effective when sublethal doses of β-lactam antibiotics were added to the bacterial cultures (17). Moreover, bacteria are more resistant against the bactericidal action of group IIA sPLA2 in the stationary phase than in the logarithmic (growth) phase, suggesting that these microorganisms are more susceptible to sPLA2 when they are dividing (17). Overall, bacterial cell wall components outside of the phospholipid membrane seem to provide a barrier for the access of sPLA2s to the phospholipid membrane surface.

The bactericidal action of group IIA sPLA2 against Gram-negative bacteria is more complex than its action against Gram-positive bacteria. Group IIA sPLA2 is bactericidal against *E. coli* by acting synergistically with the bactericidal/permeability-increasing protein (BPI), an antimicrobial protein produced by neutrophils (16). Through its interaction with bacterial lipopolysaccharide, BPI perturbs the capsules of Gram-negative bacteria and enables phospholipolysis of the bacterial phospholipid membrane by group IIA sPLA2 (18).

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[1] The abbreviations used are: sPLA2, secreted phospholipase A_2; BPI, bactericidal/permeability-increasing protein; CFU, colony forming units.

Group IIA sPLA2 enhances the action of complement components against *E. coli* and thereby increases the bacterial killing capacity of complement (19).

The fact that human group IIA sPLA2 (hGIIA)[2] has potent bactericidal properties raises the possibility that other mammalian sPLA2s are lethal to bacteria *in vitro*. Recent studies have shown that rat group V sPLA2 has bactericidal activity *in vitro* (20). We have recently developed overexpression systems that provide fully native mammalian groups I, II, V, X, and XII sPLA2s, thus permitting the bactericidal properties of these enzymes to be explored. We have also carried out a systematic study of the role of surface cationic residues (Lys and Arg) of hGIIA in supporting the bactericidal activity of this enzyme.

EXPERIMENTAL PROCEDURES

Materials—The hGIIA-N1A mutant of human group IIA sPLA2 (as well as all hGIIA-N1A mutants) was obtained by bacterial expression and *in vitro* refolding as described (21–23). Mutation of asparagine 1 to alanine allows removal of the initiator methionine. The specific activity of hGIIA-N1A using a fluorimetric assay (24) is virtually identical to that measured for wild type hGIIA obtained from a Chinese hamster ovary cell expression system (25). All hGIIA-N1A mutants were prepared using the QuikChange kit from Stratagene. The entire coding region of all mutant plasmids was sequenced. The sPLA2s mGIID, mGIIE, hGIIE, mGIIF, hGIIF, hGX, and hGXII were obtained by bacterial expression and *in vitro* refolding as described (10, 26–29). The preparation of recombinant mGIB, hGIB, mGIIA, mGIIC, mGV, hGV, and mGX will be described elsewhere. All sPLA2s were judged to be >98% pure by SDS-PAGE analysis. The experimentally determined masses of all sPLA2s were determined by electrospray mass spectrometry, and observed values agreed with theoretical values to within 1 atomic mass unit in all cases, indicating that all disulfide bonds are fully formed. Specific activities of hGIIA and its charge reversal mutants on 1-palmitoyl-2-pyrenedecanoyl-phosphatidylmethanol vesicles were measured using the fluorimetric procedure (24).

Bactericidal Studies—The bactericidal effects of sPLA2s were tested against *Staphylococcus aureus* (ATCC 25923), *L. monocytogenes* (RHD 1343), and *Escherichia coli* (ATCC 25922) (provided by the Finnish National Public Health Institute, Turku) and *B. subtilis* (IS 230) (12). To test the bactericidal effects of sPLA2s, dilutions of 100, 10, and 1 μg/ml in sterile water were prepared for each recombinant sPLA2 enzyme. Because of the small amount of enzyme available, only 10 and 1 μg/ml dilutions were prepared for hGIIE, hGIIF, mGIB, mGIIF, and mGX sPLA2s. Because of poor solubility in distilled water, hGV and mGV sPLA2s were first dissolved in a mixture of 50% acetonitrile and 0.1% trifluoroacetic acid in sterile water. The resulting solution contained 500 μg/ml sPLA2 and was diluted with sterile water to give the dilutions mentioned above. The assay of the bactericidal activity was performed as described previously (20). Briefly, 20 μl of bacterial suspension containing $\sim 5 \times 10^5$ bacteria in Hepes buffer (20 mmol/liter Hepes, 2.0 mmol/liter Ca^{2+}, 10 mg/ml bovine serum albumin, pH 7.4) was added to 20 μl of sPLA2 suspension. A mixture of 20 μl of bacterial suspension and 20 μl of sterile saline served as a sPLA2 negative control. The resulting solutions thus contained 50, 5, 0.5, and 0 μg/ml sPLA2. The sPLA2 negative control for hGV and mGV contained the same amount of acetonitrile and trifluoroacetic acid as the 50 μg/ml sPLA2 solutions. The solutions were incubated with bacteria at 37 °C with shaking at 240 rpm. Samples were taken at 20, 60, and 120 min and plated on brain heart infusion agar and grown for 24 h to measure colony-forming units (CFU). The bactericidal tests were performed twice on each bacterium and enzyme, and the results are given as mean values of duplicate determinations.

Studies of the bactericidal effect of hGIIA mutants on *B. subtilus* and *S. aureus* and release of [^{14}C]oleic acid from [^{14}C]oleic acid-labeled bacteria were carried out as described (12). Bacteria were incubated with sPLA2 for 90 min at 37 °C prior to the analysis of CFU and phospholipid hydrolysis.

RESULTS

Bactericidal Properties of Recombinant Human and Murine sPLA2s—The bactericidal effects of recombinant human and murine sPLA2s toward two Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) and against the Gram-negative bacterium *E. coli* were tested by measuring the number of CFU after incubating live bacteria with various concentrations of sPLA2 for various times (see "Experimental Procedures" and the legends to Figs. 1 and 2). Results are summarized in Figs. 1 and 2.

hGIB (Fig. 1*A*), mGIB (Fig. 1*B*), hGIIF (Fig. 2*D*), and mGIIF (Fig. 2*E*) displayed poor bactericidal activity against all three bacteria. Some of these sPLA2s displayed modest bactericidal activity only at the highest concentration of sPLA2 tested (50 μg/ml). For example, 50 μg/ml hGIB sPLA2 killed 70% of *L. monocytogenes* in 2 h but was ineffective against the other bacteria tested.

Very effective Gram-positive bactericidal activity was observed for hGIIA, mGIIA, and mGIID (Fig. 1, *C* and *D*, and Fig. 2*A*). Even at the lowest concentration tested (0.5 μg/ml), these enzymes killed over 99% of *L. monocytogenes* and *S. aureus*. All three sPLA2s showed poor bactericidal activity against *E. coli*. hGIID was not tested because of the difficulty in refolding this protein from inclusion bodies produced in *E. coli*.[3]

mGIIE, hGIIE, and mGIIC displayed intermediate potency as bactericidal agents against Gram-positive bacteria (Fig. 1*E* and Fig. 2, *B* and *C*). All three displayed measurable bactericidal activity against *L. monocytogenes* and *S. aureus* at 5 μg/ml. mGIIE killed over 99% of *L. monocytogenes* and *S. aureus* at 50 μg/ml. hGIIE was not tested at this highest concentration because sufficient amounts were not available. Neither enzyme was bactericidal against *E. coli*.

Both hGV (Fig. 3*A*) and mGV (Fig. 3*B*) were bactericidal against Gram-positive bacteria. At the concentration of 50 μg/ml, the enzymes killed 100% of *L. monocytogenes* and 90% of *S. aureus* in 2 h. At the concentration of 5 μg/ml, hGV killed ~70% of *L. monocytogenes* and 50% of *S. aureus* in 2 h. At a concentration of 5 μg/ml, mGV had no bactericidal effect against *S. aureus* but killed 50% of *L. monocytogenes*. Neither enzyme was bactericidal against *E. coli*.

At a concentration of 50 μg/ml, human hGX killed 99.9% of *L. monocytogenes* and 98% of *S. aureus* in 2 h (Fig. 3*C*). Because of the small amount of enzyme available, only concentrations of mGX up to 5 μg/ml mGX were tested (Fig. 3*D*). At this concentration, both human and murine enzymes showed similar bactericidal effects against Gram-positive bacteria. Neither enzyme was bactericidal against *E. coli*.

hGXII sPLA2 was clearly bactericidal against all three bacteria tested when tested at a high concentration (Fig. 3*E*). At a concentration of 50 μg/ml, the enzyme killed 99.9% of *L. monocytogenes* and *S. aureus* and 94% of *E. coli* in 2 h. mGXII was not available for testing, but its sequence is 94% identical to that of hGXII.

Bactericidal Studies with hGIIA Mutants—Because hGIIA and mGIIA are the most potent bactericidal sPLA2s toward Gram-positive bacteria among the human and murine sPLA2s studied, we carried out a detailed structure/function analysis of the bactericidal properties of hGIIA using a panel of mutants. We hypothesized that the highly basic nature of hGIIA (calculated pI value of 9.3) allows it to penetrate the highly anionic cell wall of Gram-positive bacteria to gain access to the outer phospholipid membrane. hGIIA contains 22 lysine and arginine residues scattered over its entire surface. As noted previously, these cationic residues are spatially segregated into eight clusters containing 1–5 basic residues (22). The largest cluster contains Lys-53, Arg-54, Lys-57, and Arg-58 located on the face of hGIIA away from the putative membrane binding

[2] A comprehensive abbreviation system for the various mammalian sPLA2s is used. Each sPLA2 is abbreviated with a lowercase letter indicating the sPLA2 species (m and h for murine and human, respectively) followed by uppercase letters identifying the sPLA2 group (GIB, GIIA, GIIC, GIID, GIIE, GIIF, GV, GX, and GXII).

[3] M. H. Gelb and G. Lambeau, unpublished observations.

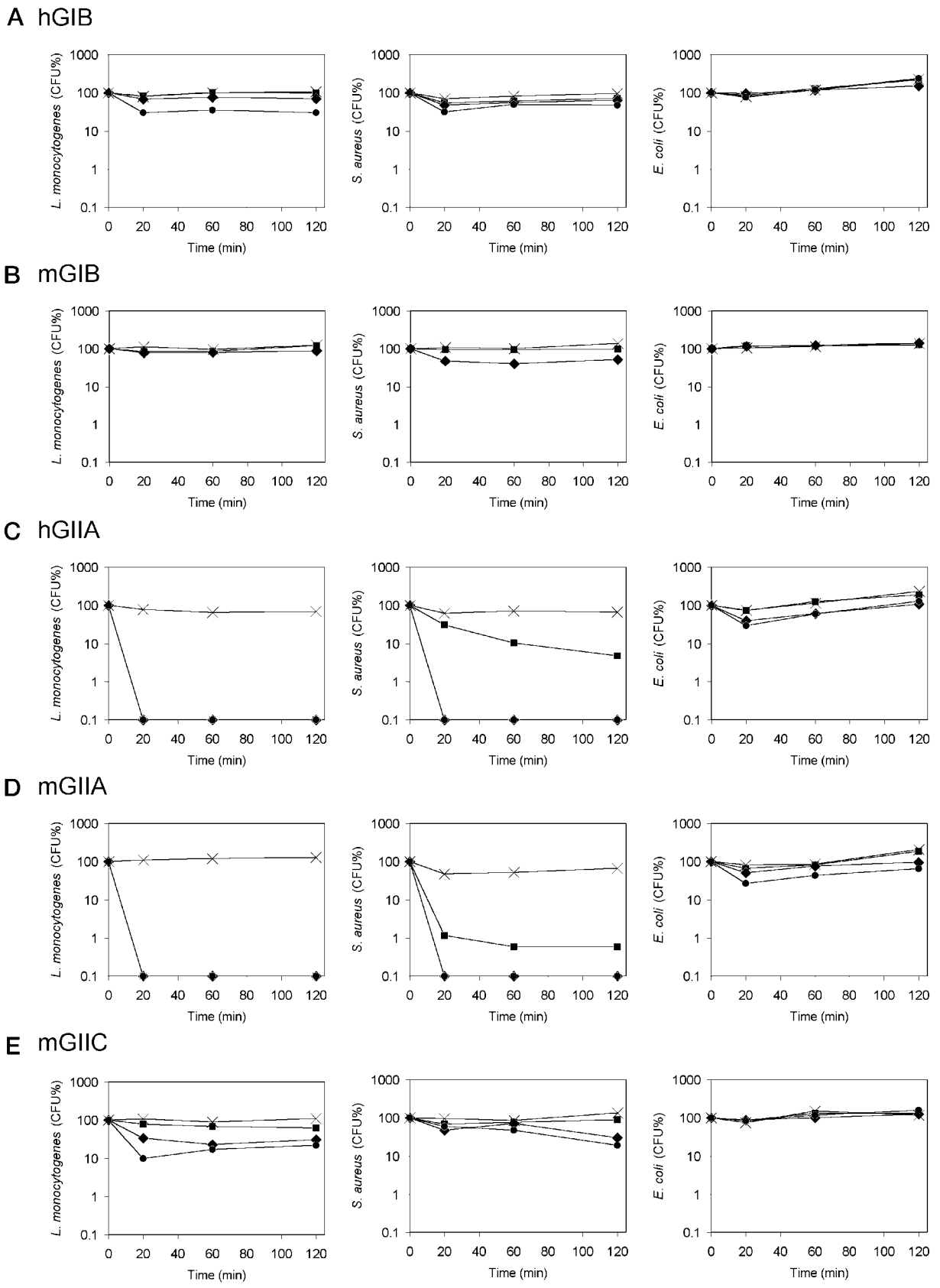

FIG. 1. ***In vitro* bactericidal activity of human and murine sPLA2s.** Samples of bacterial suspensions were taken after incubation with sPLA2s at various concentrations for 20, 60, and 120 min and thereafter cultured on agar. CFU were counted after 24 h. *A*, hGIB; *B*, mGIB; *C*, hGIIA; *D*, mGIIA; *E*, mGIIC. The results shown in the figure are means of two independent tests. The sPLA2 concentrations were as follows: × = 0 μg/ml sPLA2, ■ = 0.5 μg/ml sPLA2, ♦ = 5 μg/ml sPLA2, ● = 50 μg/ml sPLA2.

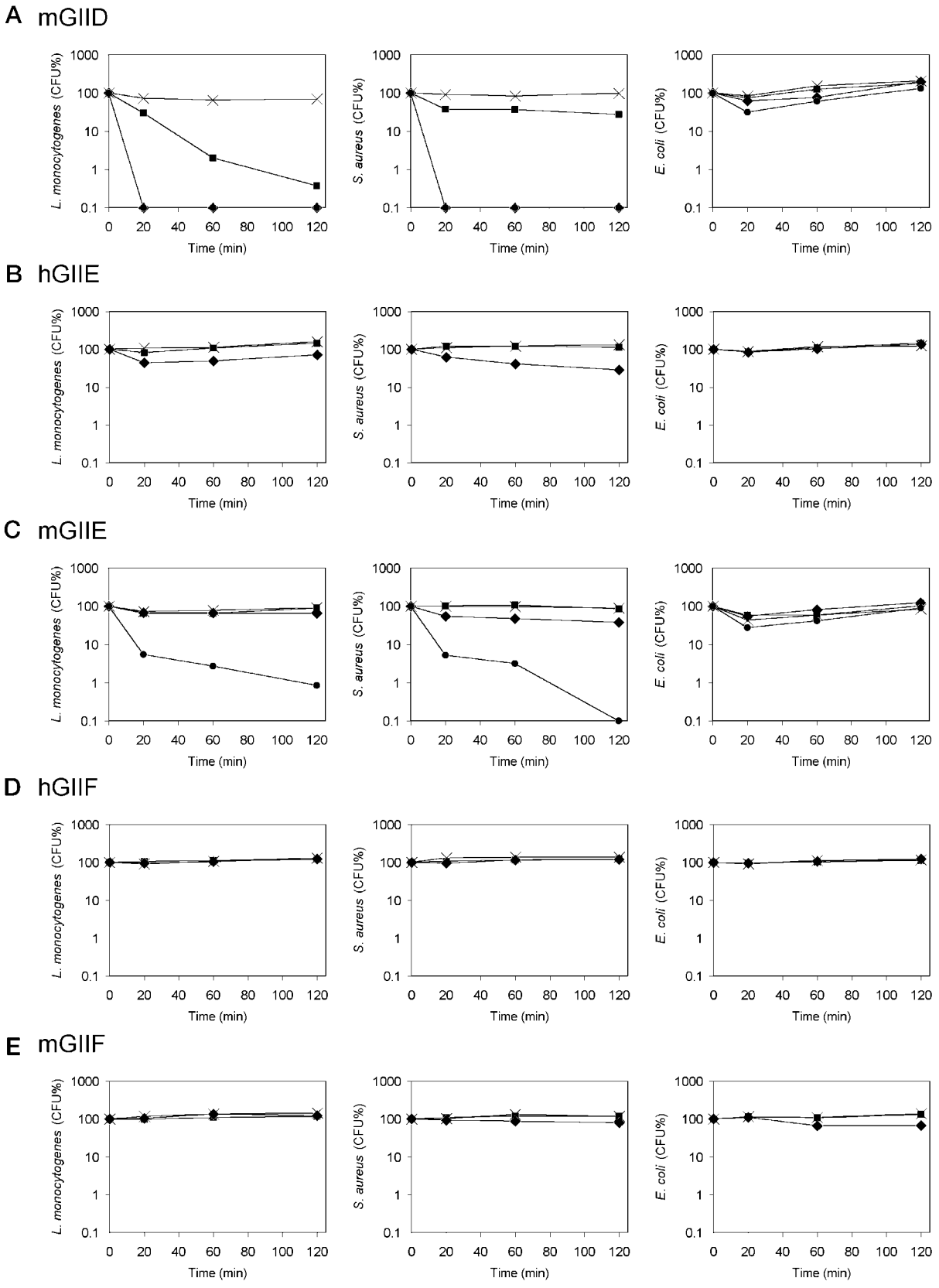

FIG. 2. ***In vitro* bactericidal activity of human and murine sPLA2s.** Details are the same as described in the legend for Fig. 1. *A*, mGIID; *B*, hGIIE; *C*, mGIIE; *D*, hGIIF; *E*, mGIIF. The results shown in the figure are means of two independent tests. The sPLA2 concentrations were as follows: × = 0 μg/ml sPLA2, ■ = 0.5 μg/ml sPLA2, ♦ = 5 μg/ml sPLA2, ● = 50 μg/ml sPLA2.

surface (see Fig. 1 of Ref. 23 for a structural depiction of these basic residues). Three charge reversal mutations were introduced into this cluster (K53E/R54E/R58E). The double- and triple-site mutants K38E/K116E, R7E/K10E/K16E, and K74E/K87E/R92E contain charge reversal in clusters that lie on the putative membrane binding surface of hGIIA. The double mu-

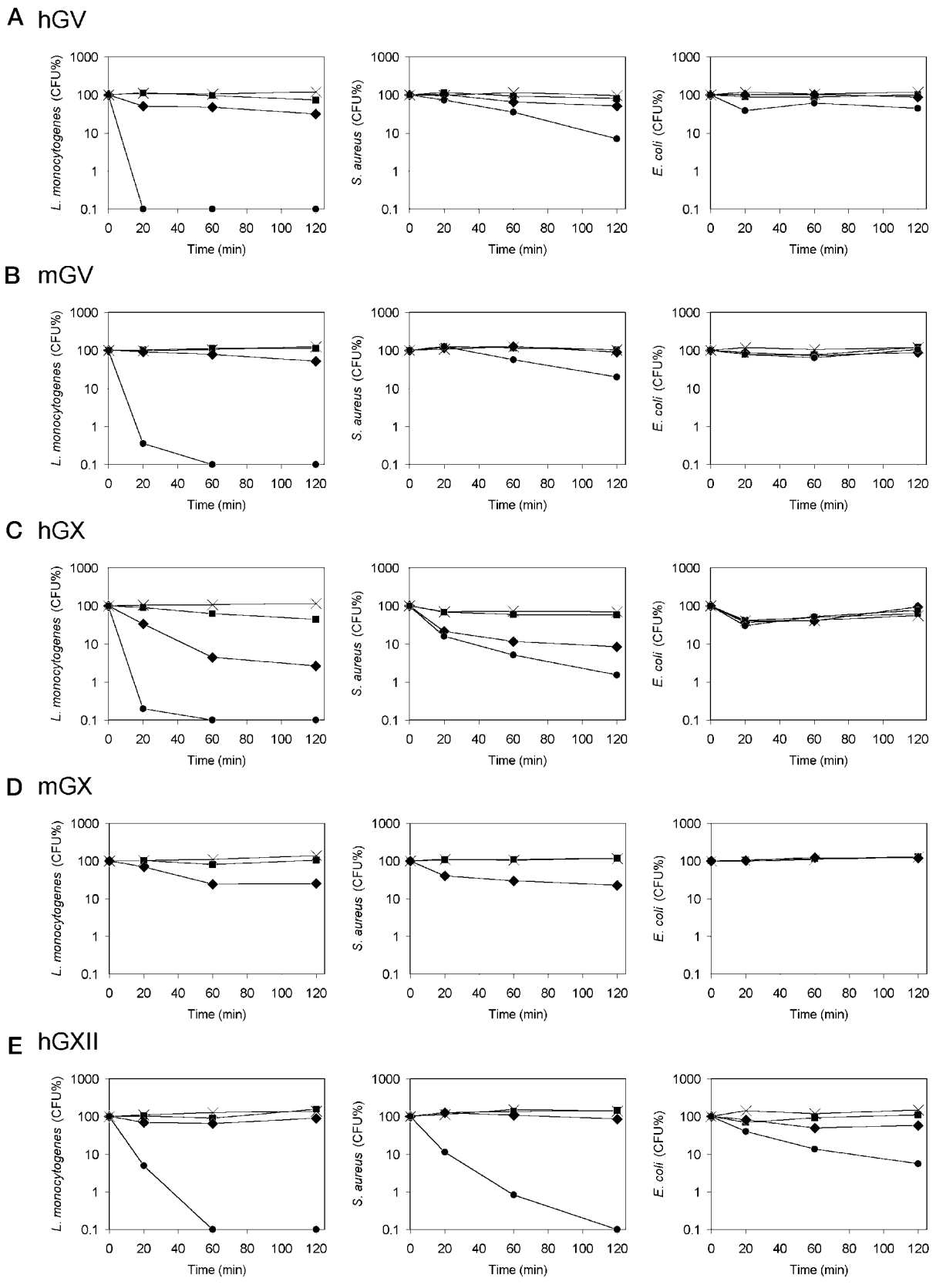

FIG. 3. ***In vitro*** **bactericidal activity of human and murine sPLA2s.** Details are the same as described in the legend for Fig. 1. *A*, hGV; *B*, mGV; *C*, hGX; *D*, mGX; *E*, hGXII. The results shown in the figure are means of two independent tests. The sPLA2 concentrations were as follows: × = 0 μg/ml sPLA2, ■ = 0.5 μg/ml sPLA2, ♦ = 5 μg/ml sPLA2, ● = 50 μg/ml sPLA2.

tants K110E/K115E and K124E/R127D are in separate clusters that are close to the putative membrane binding surface. Some of these charge reversal cluster mutations were combined into the same protein (K53E/R54E/R58E/K124E/R127D and K38E/K110E/K115E/K116E), and some mutants were prepared that contain a smaller number of charge reversal muta-

TABLE I
Bactericidal and phospholipid hydrolysis properties of wild type and mutant hGIIA sPLA2s

hGIIA phospholipase A_2	Phosphatidyl-methanol vesicle hydrolysis	CFU *B. subtilis* EC_{50}[a]	Phospholipid hydrolysis *B. subtilis*[b]	Phospholipid hydrolysis *B. subtilis* protoplasts[c]	CFU *S. aureus* EC_{50}[a]	Phospholipid hydrolysis *S. aureus* autoclaved[a]
	% WT	*% WT*	*% WT*	*% WT*	*% WT*	*% WT*
Wild type	100	100[d]	100, 100, 100	100	100[d]	100
R92E	124	18	20, 50, 100	800	12	90
K87E	109	25	25, 50, 100	800	10	100
K74E	133	20	23, 45, 90	700	10	100
R7E	52	20	15, 45, 100	700	4	90
K110E/K115E	111	5	6, 12, 80	150	1	50
K38E/K116E	103	3	2, 6, 15	150	1	40
K10E/K16E	100	1	1.5, 3, 15	700	<0.1	40
R7E/K10E	129	1.5	1.5, 5, 20	20	<0.1	30
K124E/R127D	60	1	1, 5, 25	100	<0.1	90
K53E/R54E/R58E	75	10	10, 20, 110	900	<0.1	90
K74E/K87E/R92E	116	<0.1	<0.1, 0.8, 10	300	<0.1	30
R7E/K10E/K16E	92	<0.1	<0.1, 1, 10	30	<0.1	5
K38E/K110E/K115E/K116E	70	<0.1	<0.1, <0.1, 3	120	<0.1	1
K53E/R54E/R58E/K124E/R127D	60	<0.1	<0.1, <0.1, 5	200	<0.1	0.8

[a] 10^6 bacteria/ml.
[b] 10^6, 10^7, 10^8 bacteria/ml.
[c] 10^7 bacteria/ml.
[d] Values of EC_{50} for killing of *B. subtilis* and *S. aureus* by wild type hGIIA sPLA2 are 5 and 250 ng/ml, respectively.

tions (R7E/K10E, K10E/K16E, R7E, K74E, K87E, R92E).

As shown in Table I, the specific activity of wild type hGIIA and all charge reversal mutants are similar (52–133% of wild type) when measured with a fluorimetric assay using 1-palmitoyl-2-pyrenedecanoyl-phosphatidylmethanol vesicles. This indicates that all mutants were structurally intact and that all mutants bound well to anionic phosphatidylmethanol vesicles.

All of these mutant hGIIA proteins were tested for their bactericidal activity on Gram-positive bacteria and for their ability to release radiolabeled oleic acid from bacterial membranes. Fig. 4 shows the *B. subtilis* bactericidal dose-response curves for wild type hGIIA and six charge reversal mutants. Table I lists the *B. subtilis* bactericidal activity of all mutants relative to that of wild type hGIIA. There is a dominant and general trend that bactericidal activity decreases as the amount of positive charge on hGIIA is decreased regardless of whether cationic residues on or not on the putative membrane binding site are mutated. A secondary effect is also apparent. Lysines and arginines on the putative membrane binding surface appear to be modestly more important for supporting bactericidal activity than are residues that are not on the membrane binding surface. For example, the double mutant R7E/K10E is less potent at killing *B. subtilis* than is the triple mutant K53E/R54E/R58E. It is significant that K53E/R54E/R58E is considerably less potent at killing *B. subtilis* than is wild type hGIIA despite the fact that these charge reversal mutations are on the opposite face of the enzyme from the membrane binding surface. Pig pancreatic sPLA2 displays no bactericidal activity (not shown) as reported previously (30, 31).

Table I lists the relative EC_{50} (concentrations eliciting 50% effect) values for the bactericidal activity of all of the charge reversal hGIIA mutants acting on the Gram-positive bacterium *S. aureus*. As reported previously (12), wild type hGIIA is about 50-fold less potent as a bactericidal agent against *S. aureus* than against *B. subtilis* (EC_{50} values of 250 and 5 ng/ml toward *S. aureus* and *B. subtilis*, respectively). The trend for the charge reversal mutants is similar to that seen with *B. subtilis*, *i.e.* bactericidal activity falls as the number of lysine and arginine residues is mutated to anionic residues. Bactericidal potency on *S. aureus* is more sensitive to charge reversal mutagenesis than on *B. subtilis* (Table I). For example, even single site charge reversal mutants reduce bactericidal potency about 10-fold, and the triple mutant K53E/R54E/R58E displays no bactericidal activity on *S. aureus* when tested up to 1 μg/ml

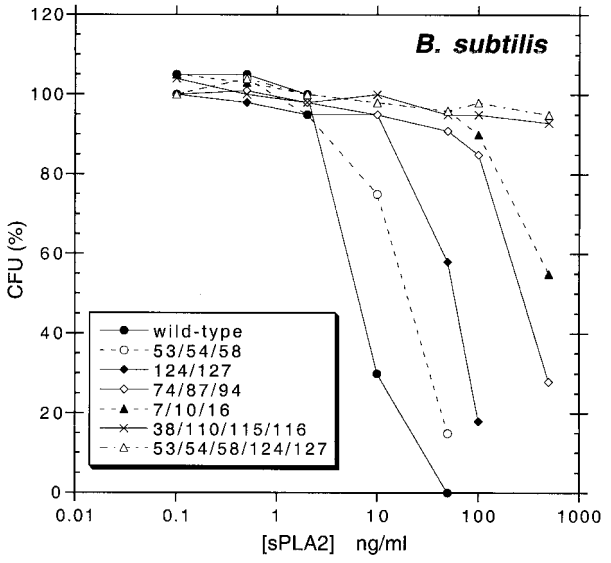

FIG. 4. ***B. subtilis* bactericidal activity of wild type and mutant hGIIA sPLA2s.** Live *B. subtilis* (10^6/ml) was incubated for 90 min at 37 °C with the indicated concentration of sPLA2, and the number of surviving bacteria (CFU) was quantified as described under "Experimental Procedures." Numbers are expressed as a percentage of CFU measured in the absence of sPLA2.

even though this hGIIA protein has a fully native membrane binding surface.

Bacterial Membrane Hydrolysis Studies with hGIIA Mutants—The ability of the charge reversal mutants to liberate oleic acid from bacterial membrane phospholipids was also studied. Dose-response curves are shown in Fig. 5 for the action of hGIIA and six charge reversal mutants on *B. subtilis*, and Table I compares the fatty acid release by all mutants. Incubation of radiolabeled bacteria with increasing amounts of wild type hGIIA leads to a maximum release of about 60% of the total ^{14}C incorporated in bacteria. This is close to the expected amount (~50%) if only the phospholipids in the extracellular leaflet of the bilayered membrane (that which is accessible to sPLA2) become hydrolyzed. The ability of the hGIIA mutants to release oleic acid from membrane phospholipids correlates well with the relative bactericidal activity (compare Figs. 3 and 4 and the data in Table I).

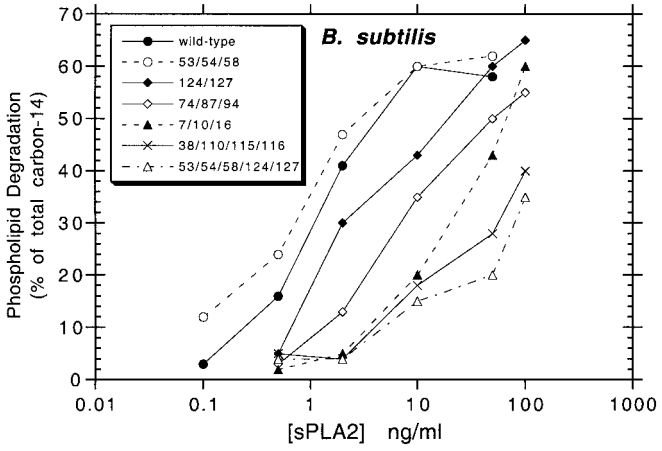

FIG. 5. ***B. subtilis* membrane phospholipid hydrolysis by wild type (*WT*) and mutant hGIIA sPLA2s.** Live *B. subtilis* (10^6/ml) were labeled with [^{14}C]oleic acid. Labeled bacteria were incubated for 90 min at 37 °C with the indicated concentrations of sPLA2, and liberated [^{14}C]oleic acid was quantified as described under "Experimental Procedures." Numbers are expressed as a percentage of the total ^{14}C in bacteria that is liberated after incubation with sPLA2.

We hypothesized that the charge reversal mutants are less capable of hydrolyzing the membrane of intact *B. subtilis* because of poor penetration into the anionic cell wall. If so, the effect of charge reversal mutagenesis may be partially overcome by increasing the concentration of bacteria in the reaction mixture with hGIIA, which will drive the equilibrium between free and bacteria-bound sPLA2 in favor of the latter (mass action). As shown by the data in Table I, this is indeed the case. As the concentration of *B. subtilis* in the reaction mixture with hGIIA is increased from 10^6/ml to 10^8/ml, the ability of the charge reversal mutants to hydrolyze bacterial phospholipids is fully restored for the single site mutants and for the triple mutant K53E/R54E/R58E and is partially restored for the other multisite mutants.

We also studied the ability of hGIIA and its charge reversal mutants to release oleic acid from membrane phospholipids of *B. subtilis* protoplasts, which contact an intact phospholipid membrane but lack a cell wall, and the results are summarized in Table I. With protoplasts, a dramatically different behavior is seen compared with intact bacteria in that all of the proteins showed very similar ability to hydrolyze membrane phospholipids. With 10^7 bacteria/ml, the maximum span in relative efficiency of protoplast membrane phospholipid hydrolysis for wild type *versus* mutant hGIIA is 5-fold, which compares with the maximum span of >1,000-fold for the bactericidal activity of these proteins against 10^7/ml live *B. subtilis* (Table I). Note that some hGIIA mutants display a higher specific activity than wild type for the hydrolysis of protoplast phospholipids. For example, K53E/R54E/R58E is 9-fold more active than wild type hGIIA in this assay.

When *S. aureus* was autoclaved to disrupt its cell wall and expose its membrane phospholipids, all mutants were considerably more effective at liberating oleic acid (Table I). With 10^6 bacteria/ml, the maximum span in relative efficiency of autoclaved membrane phospholipid hydrolysis for wild type *versus* mutant hGIIA is 125-fold, which compares with the maximum span of >1,000-fold for the bactericidal activity of these proteins against 10^6/ml live *S. aureus* (Table I). In contrast to results with *B. subtilus* protoplasts, none of the hGIIA mutants display specific activity on autoclaved *S. aureus* membranes higher than that of wild type.

DISCUSSION

Bactericidal Activity of Murine and Human sPLA2s—With the availability of all of the human and murine groups I, II, V, X, and XII sPLA2s in recombinant form (except hGIID and mGXII), it has been possible to determine the relative bactericidal potencies of these enzymes against two Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) and against the Gram-negative bacterium *E. coli*. The Gram-positive bactericidal potencies of mammalian groups I, II, V, X, and XII differ dramatically (Figs. 1 and 2). Among human sPLA2s, the rank order potency for bactericidal activity against *L. monocytogenes* is hGIIA > hGX > hGV > hGXII > hGIIE > hGIB, hGIIF. The mouse sPLA2s show the same trend except for the fact that mGX is significantly less bactericidal than hGX against *L. monocytogenes* (mGIIA > mGIID > mGV > mGIIE > mGX > mGIB, mGIIF). Similar trends are seen for bactericidal action against *S. aureus* for both human and murine sPLA2s.

The cell wall of Gram-positive bacteria contains a dense peptidoglycan array, which bears a highly anionic charge due to the presence of phosphate diester units of lipoteichoic acid. Because the sPLA2 must traverse the highly anionic cell wall of Gram-positive bacteria to reach the phospholipid membrane target, the degree of positive charge on the surface of the enzyme could be important for bactericidal activity. Among the human sPLA2s, relative pI values may partially explain the relative Gram-positive bactericidal potencies. hGIIA is the most basic human sPLA2 (pI ~9.4; all stated pI values are calculated values) and is the most potent bactericidal enzyme. hGIIF on the other hand is an acidic protein (pI ~4.74) and is devoid of bactericidal activity. hGIIE and hGV display bactericidal activity with intermediate potency and have comparable values of pI that are less than that for hGIIA (8.5 and 8.7, respectively). Other factors besides basicity are important for bactericidal activity. Even though the pI values of hGV and hGIIE are similar, the bactericidal potency of hGV is significantly higher than that of hGIIE. This may be due to the fact that the *in vitro* specific activity for the hydrolysis of a variety of phospholipid vesicles is ~50-fold higher for hGV than for hGIIE.[4] hGXII is significantly more bactericidal against Gram-positive bacteria than is hGIB, yet the pI of hGXII is ~6.3 *versus* ~8.0 for hGIB. The other exception to the trend between pI and bactericidal potency is seen for hGX, which is a very acidic sPLA2 (pI ~5.1) and is the second most potent human sPLA2 against Gram-positive bacteria. The *in vitro* specific activity for the hydrolysis of a variety of phospholipid vesicles by hGX is ~50-fold higher than that for hGXII (28, 29), which may explain why hGX is more bactericidal than hGXII despite the fact that the pI for the former is less than that of the latter. However, the *in vitro* vesicle hydrolysis specific activities of hGX and hGV are comparable,[4] hGX is considerably more acidic than hGV (pI ~5.1 *versus* 8.7), and yet hGX is more bactericidal than hGV. Clearly, there is no simple algorithm that can predict the relative bactericidal potencies of the human sPLA2s against Gram-positive bacteria. One can anticipate that the arrangement of basic residues on the surface of the enzyme in addition to the overall charge on the protein may be most important for bactericidal potency. Additional support for this comes from the studies of Weiss *et al.* (30) in which the poorly bactericidal porcine pancreatic group IB sPLA2 was converted to a potent bactericidal agent against BPI-treated *E. coli* by site-directed mutagenesis. Increasing the net positive charge on the porcine enzyme from −1 to +4 increased bactericidal potency, but most of this increase occurred when a single residue, Ser-7, was mutated to Arg or Lys, and the mutant with a +4 overall charge lacked bactericidal activity when Ser-7 was not altered.

[4] M. H. Gelb, unpublished observations.

Among the murine sPLA2s, the relative bactericidal potency against Gram-positive activity follows the relative pI values (9.3 for mGIIA, 8.9 for mGIID, 8.4 for mGV, 8.2 for mGIIE, 5.4 for mGX, 8.0 for mGIB, 4.7 for mGIIF) with the exception of mGIB, which is poorly bactericidal despite its relative high pI value. The *in vitro* vesicle hydrolysis specific activity of mGIIE is ~10-fold higher than that for hGIIE, which may explain why mGIIE is ranked higher than hGIIE as a bactericidal agent.

hGIIA and mGIIA sPLA2s may have a significant role *in vivo* in bacterial infections by killing bacteria present in the circulation. We have shown that overexpression of hGIIA in transgenic mice protects the animals against *S. aureus* (32) and *E. coli* (33) infections. The principal Gram-positive bactericidal component of human tears is hGIIA (15). Group IIA sPLA2, or a closely related sPLA2, is responsible for the Gram-positive (*S. aureus* and others) bactericidal activity of the cell-free ascitic fluid of a sterile inflammatory peritoneal exudate elicited in rabbits (12). Furthermore, the concentration of sPLA2 in this fluid is more than sufficient to kill Gram-positive bacteria *in vitro*. Group IIA sPLA2 is abundantly present in the circulation in inflammatory diseases (34) and may have a significant role in bacterial infections by killing bacteria (13, 32). Our preliminary data show that the presence of hGIIA almost completely accounts for the bactericidal activity of human acute phase serum.[5] The results of the present study showing that hGIIA and mGIIA are the most potent Gram-positive bactericidal enzymes among groups I, II, V, X, and XII sPLA2s support their proposed role in bactericidal function *in vivo*.

Group V sPLA2 has been shown to be present in murine macrophages and mast cells and to function in inflammatory responses (7, 8, 35, 36). Recent studies show that hGV is the major sPLA2 secreted from circulating neutrophils stimulated with the bacterial-derived peptide *N*-formyl-Met-Leu-Phe.[6] It is possible that group V sPLA2 may exert its bacterial potency in mammalian phagocytes. In addition, group V sPLA2 is expressed in the mouse eye (29), raising the possibility that this enzyme functions as a bactericidal agent of murine tears.

The bactericidal action of hGIIA against Gram-negative bacteria is more complex than its action against Gram-positive bacteria. hGIIA is bactericidal against *E. coli* only when it acts synergistically with the BPI, an antimicrobial protein produced by polymorphonuclear leukocytes (18). Through its interaction with bacterial lipopolysaccharide, BPI perturbs the lipopolysaccharide capsule of Gram-negative bacteria and enables phospholipid hydrolysis of the bacterial phospholipid membrane by hGIIA. Among all the murine and human sPLA2s tested against *E. coli*, only hGXII displayed bactericidal activity against this Gram-negative bacterium in the absence of BPI but with weak potency. Apparently even the highly cationic sPLA2s such as hGIIA, mGIIA, and mGIID cannot traverse the anionic and dense cell wall of *E. coli* in the absence of BPI.

Human pancreatic juice has been reported to display bactericidal activity against a number of bacteria including *E. coli* (37). Based on the poor bactericidal activity of hGIB (Table I) and porcine group IB (pancreatic) sPLA2s (30), it seems likely that other pancreatic proteins are responsible for the bactericidal activity of this biological fluid. The presence of other sPLA2s in pancreatic juice besides group IB needs to be considered because mRNA for non-pancreatic sPLA2s have been detected in total RNA isolated from whole pancreas (27, 29). Furthermore, mice containing a disrupted *mGIB* gene display apparently normal phospholipid digestion, supporting the presence of other sPLA2s in pancreatic juice (38).

Bactericidal Activity of Wild Type and Mutant hGIIA sPLA2s—Bacterial killing by rabbit group IIA sPLA2 (or a closely related sPLA2) is abrogated by alkylation of its active site histidine with *p*-bromophenacylbromide or by removal of Ca^{2+}, thus arguing that phospholipase activity is required for bactericidal activity (12, 18). In the present study, it was found that the relative *B. subtilis* and *S. aureus* bactericidal potencies of hGIIA and all of the 14 charge reversal mutants studied correlates perfectly with their relative abilities to hydrolyze *B. subtilis* and *S. aureus* membrane phospholipids ([^{14}C]oleate release from [^{14}C]oleate-labeled bacteria). Pretreatment of Gram-positive bacteria with β-lactam antibiotics increased the rate of sPLA2-catalyzed phospholipid degradation and increased bactericidal potency (17). All of these results strongly argue that the Gram-positive bactericidal activity of sPLA2s is the result of membrane degradation.

On the other hand, two myotoxic sPLA2s from the venom of the *Bothrops asper* snake display bactericidal activity, and yet one of these proteins is enzymatically inactive because it contains a lysine in place of the calcium-binding aspartate ([Lys-49]enzyme) (39). This result argues that the bactericidal activity of these venom sPLA2s occurs by a mechanism distinct from that of mammalian sPLA2s. This is also supported by the fact that these venom enzymes are bactericidal against Gram-negative bacteria in the absence of BPI and against Gram-positive bacteria. Furthermore, the bactericidal activity can be mimicked by a short peptide corresponding to residues 115–129, and both peptide and protein render the bacterial membrane more permeable to hydrophobic probes (39). These results are typical of venom sPLA2s, which have evolved to contain short, contiguous amino acid segments that bind to a number of biological targets as the basis for their toxic effects.

In the present study, conversion of basic residues of hGIIA to acidic residues by site-directed mutagenesis was used to examine the features of this sPLA2 that are required for Gram-positive bactericidal activity. The dominant trend is that bactericidal activity tracks well with the overall degree of positive charge on hGIIA. The only deviation from this trend is the observation that, among triple-site mutants, K53E/R54E/R58E is more bactericidal than K74E/K87E/R92E and R7E/K10E/K16E (all three lack measurable bactericidal activity against *S. aureus*). The latter two triple-site mutants involve basic residues that are on the putative membrane binding surface of hGIIA, suggesting that these basic residues may contribute to the binding of hGIIA to the highly anionic, phosphatidylglycerol-rich bacterial membrane. The effect of location of charge reversal mutation is modest, however, compared with the effect of overall positive charge reduction on the surface of the protein. This result is consistent with our previous studies (22, 23) showing that charge reversal mutagenesis of putative membrane binding site basic residues produces only a modest reduction in affinity for anionic phospholipid vesicles. For example, the double-site mutant R7E/K10E involves residues that are almost certainly on the membrane binding surface of hGIIA because these N-terminal helix residues are located on the surface of the enzyme adjacent to the opening to the active site slot where a single phospholipid molecule must bind for catalysis. For this mutant, the reduction in binding to phosphatidylglycerol vesicles *in vitro* compared with wild type is only ~100-fold and displays high affinity for anionic vesicles (vesicle-to-aqueous layer dissociation equilibrium constant <1 μM). (22, 23). The fact that all charge reversal mutants display similar specific activity on low micromolar concentrations of

[5] J. O. Gronroos, V. J. O. Laine, and T. J. Nevalainen, submitted for publication.

[6] Degousee, N., Chomashchi, F., Stefanski, E., Singer, A., Smart, B. P., Borregaard, N., Reithmeier, R., Lindsay, T. F., Lichtenberger, C., Reinisch, W., Lambeau, G., Arm, J., Tischfield, J., and Gelb, M. H. (2002) *J. Biol. Chem.* **277,** 5061–5073.

anionic phosphatidylmethanol vesicles (Table I) also shows that all mutants have interfacial dissociation constants of <1 μM. The observation that wild type and charge reversal hGIIA mutants display similar specific activity on exposed Gram-positive bacterial membranes (*S. subtilis* protoplasts and autoclaved *S. aureus*, Table I) is consistent with the findings with phosphatidylmethanol and phosphatidylglycerol vesicles and further shows that binding to anionic membranes is not significantly impaired following conversion of basic residues to acidic ones. The fact that basic residues scattered over the entire, nearly spherical surface of hGIIA are important for bactericidal potency against Gram-positive bacteria argues that binding of the protein to anionic teichoic acid residues of the cell wall occurs via electrostatic interactions that are not dominated by any particular patch on the surface of the enzyme. This in turn allows for the possibility that hGIIA penetrates the cell wall by a processive or "rolling" motion involving multiple and reversal protein-cell wall contacts.

Our results with wild type and hGIIA are consistent with the recent studies of Buckland *et al.* (31). Disruption of the cell wall of *Micrococcus luteus* by lysozyme treatment allows the acidic porcine pancreatic and cobra venom sPLA2s to hydrolyze the bacterial membrane. Disruption of the cell wall of *S. aureus* with the specific cell wall protease lysostaphin increased the phospholipase activity of hGIIA on the bacterial membrane, indicating that the intact cell wall provides a barrier for membrane degradation even by the highly penetrating hGIIA. This result underscores the possibility that mixtures of hGIIA with cell wall-disrupting agents may have practical utility for the development of antiseptic formulations. The data in this study showing that many mammalian sPLA2s have bactericidal activity and the fact that these enzymes can co-localize in a single tissue argue that they may act in concert under pathophysiological conditions such as sepsis.

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