Bactericidal Properties of Murine Intestinal Phospholipase A_2

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Abstract

We purified a molecule from the murine small intestine that killed both *Escherichia coli* and *Listeria monocytogenes*, and identified it as intestinal phospholipase A_2 (*i*PLA_2) by NH_2-terminal sequencing and enzymatic measurements. The ability of *i*PLA_2 to kill. *L. monocytogenes* was greatly enhanced by 5 mM calcium, inhibited by EGTA and abolished after reduction and alkylation, suggesting that enzymatic activity was required for *i*PLA_2-mediated bactericidal activity. A mouse-avirulent phoP mutant, *S. typhimurium* 7953S, was 3.5-fold more susceptible to *i*PLA_2 than its isogenic virulent parent, *S. typhimurium* 14028S (estimated minimal bactericidal concentrations 12.7±0.5 μg/ml *vs.* 43.9±4.5 μg/ml, $P < 0.001$). Overall, these findings identify *i*PLA_2 as part of the antimicrobial arsenal that equips Paneth cells to protect the small intestinal crypts from microbial invasion. Because *i*PLA_2 is identical to Type 2 phospholipase A_2 molecules found in other sites, including spleen, platelets and inflammatory exudate cells, this enzyme may also contribute to antibacterial defenses elsewhere in the body. (*J. Clin. Invest.* 1995. 95:603–610.) Key words: defense mechanisms • host–parasite relations • immunity, natural • intestinal mucosa • phospholipases A

Introduction

Despite an intermittently rich content of nutrients and the repeated entry of bacteria in food and oral secretions, the small intestine normally contains very few microorganisms (1). The host factors that prevent local microbial proliferation are uncertain, but gastric acidity, epithelial cell shedding, the mechanical effects of peristalsis, and the actions of bile salts are traditionally mentioned.

Paneth cells are specialized epithelial cells located in the basal portion of intestinal crypts, subjacent to the zone of intestinal epithelial cell division. They contain and secrete lysozyme and enteric defensins ("cryptdins") that are homologous to the antimicrobial defensins found in myeloid cells (2–8). Given the strategic location of Paneth cells, their content of antimicrobial proteins and peptides, and their phagocytic (9, 10) and secretory (11, 12) abilities, these once enigmatic cells are likely to contribute to intestinal antimicrobial defenses.

During a recent purification of cryptdins from preparations enriched for murine Paneth cells, we noted the presence of larger polypeptides with very potent antimicrobial activity (see Fig. 1 *b* in reference 6). We now describe the purification of one of these molecules, and its identification as a Type 2 phospholipase A_2, a known Paneth cell constituent in rats (13) and humans (14).

Methods

Identification of antimicrobial Paneth cell components

Preparation of a Paneth cell–enriched fraction from murine small intestine was described recently (6). Briefly, small intestines were excised from fasted female adult Swiss Webster mice, quadrisected, flushed with 0.9% NaCl containing 1 mM dithiothreitol, everted over thin glass pipettes, and twice incubated for 30 min in phosphate buffered saline with 27 mM sodium citrate, pH 7.4. The exfoliated cells were combined, recovered by centrifugation (450 *g*, 10 min 4°C) resuspended in ice-cold 0.34 M sucrose, pH 7.4, disrupted in a Potter-Elvehjem homogenizer to > 90% cellular breakage. A post nuclear supernatant was prepared, centrifuged (27,000 *g*, 20 min) and its pellet was extracted with ice-cold 5% acetic acid to provide starting material for our initial studies.

This extract was chromatographed on a SynChropak GPC 100 column (Synchrom, Lafayette, IN) with 100 mM ammonium acetate-1% acetic acid in 10% acetonitrile. Each fraction was tested for antimicrobial activity against *Listeria monocytogenes* EGD by a radial diffusion assay (15), and examined by acid–urea polyacrylamide gel electrophoresis (AU-PAGE).[1] Fractions of interest were further purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on a 4.6 × 250 mm Vydac C-18 column (The Separations Group, Hesperia, CA), using a linear water-acetonitrile gradient that contained 0.1% trifluoroacetic acid (TFA) and increased by 1% acetonitrile min^{-1}. Each RP-HPLC fraction was tested for antimicrobial activity, and examined by AU-PAGE and SDS-PAGE. Fractions containing enteric defensins (cryptdins) and lysozyme were identified and reserved for other studies, and other fractions with potent antimicrobial activities were selected for further purification. The protein described in this report was initially recognized by gel overlay assays (15) against *L. monocytogenes* and we provisionally identified it as a Type 2 intestinal PLA_2 by performing NH_2-terminal microsequencing after electro-transferring it to a PVDF membrane (16).

Scaled-up purification

To obtain larger amounts of this iPLA_2, 30 g of mouse intestinal acetone powder (catalog number I-1755; Sigma Chemical Co., St. Louis, MO) was homogenized in 300 ml ice-cold 1 N HCl, stirred for 4 h in the cold, and centrifuged at 27,000 *g* for 20 min at 4°C. The insoluble residue was re-extracted as above, and the combined supernatants were ultrafiltered sequentially through YM 30 and YM 5 membranes (Ami-

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1. *Abbreviations used in this paper:* AU-PAGE, acid-urea PAGE; *i*PLA_2, intestinal phospholipase A_2; eMBC, estimated minimal bactericidal concentration; PLA_2, phospholipase A_2; PVDF, polyvinylidene difluoride; RP-HPLC, reversed phase-HPLC; TFA, trifluoroacetic acid.

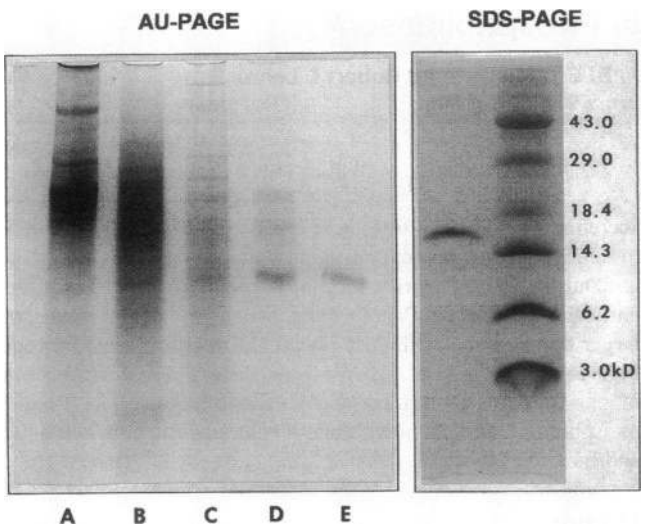

Figure 1. Purification of *i*PLA2 from mouse intestine acetone powder. AU-PAGE gels illustrating the stages of purification. Lane *A*, crude 1N HCl extract (20 μg protein); lane *B*, YM30 filtrate/YM 5 retentate (20 μg protein); Lane *C*, pooled PLA_2-containing fractions after RP-HPLC (10 μg protein); lane *D*, PLA_2-containing fractions after cation exchange HPLC (4 μg protein); lane *E*, purified PLA_2 after continuous AU-PAGE (1 μg protein). The inset on the right shows a silver stained Tricine SDS-PAGE of the purified material.

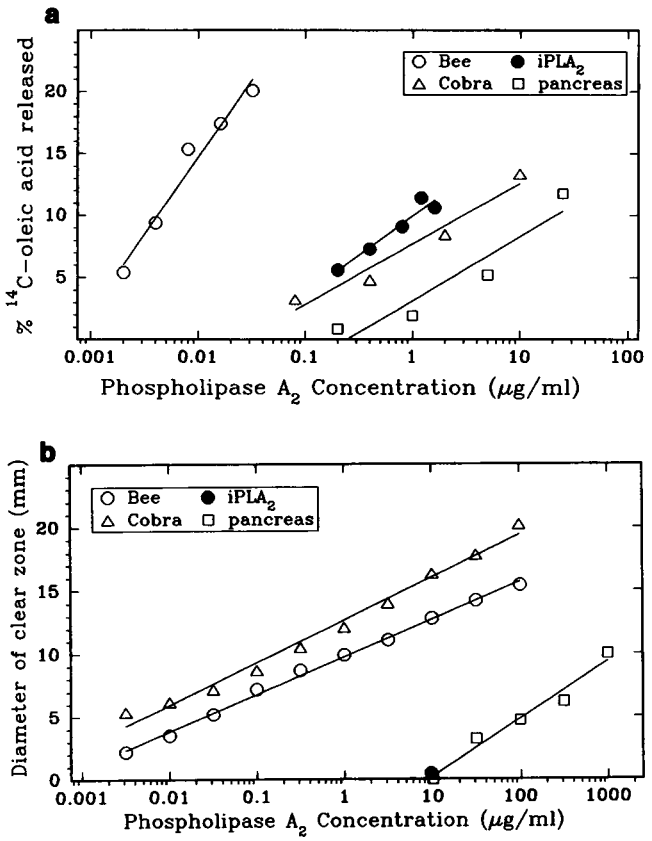

Figure 2. Enzyme activity. Purified murine *i*PLA_2 was compared with PLA_2s obtained from cobra venom (*Naja mocambique*) and bovine pancreas, with autoclaved [^{14}C]oleic acid–labeled *E. coli* (*a*) or egg yolk (*b*) as substrate.

con, Inc., Beverly, MA). The YM 30 filtrate/YM 5 retentate was purified by RP-HPLC on a 10 × 250 mm Vydac C-18 column with a linear water-acetonitrile gradient that contained 0.1% TFA. Each fraction was examined by AU-PAGE and tested for PLA_2 enzyme activity with [^{14}C]oleic acid–labeled *E. coli*, as described below. Fractions with phospholipase activity were concentrated, dialyzed against 50 mM phosphate buffer, pH 6.55, chromatographed on a PolyCat-A cation-exchange HPLC column (PolyLC, Columbia, MD), eluted with a gradient of NaCl and desalted by RP-HPLC. After concentration, the PLA_2 was further purified by preparative AU-PAGE (17) on a Model 491 Prep Cell (Bio-Rad Laboratories, Hercules, CA) that contained an 8 cm high, 12.5% acrylamide gel. Final purification was accomplished by RP-HPLC on a 4.6 × 250 mm Vydac C-18 column. From 30 g of acetone powder, we recovered ~ 120 μg of highly purified PLA_2 plus an additional 180 μg of > 95% pure PLA_2. The highly purified preparation was used for all studies reported here, unless otherwise noted.

Phospholipase A_2 assays

Bacteria. Autoclaved [^{14}C]oleic acid–labeled *E. coli* ML-35 were prepared essentially as described by Elsbach and Weiss (18), and their specific activity was adjusted by adding autoclaved native bacteria, so that 50,000 cpm corresponded to approximately 2.5×10^8 total bacteria. Samples were incubated for 15 min at 37°C with ^{14}C-*E. coli* (50,000 cpm) in 250 μl of 250 mM Tris buffer that contained 10 mM $CaCl_2$ and 1 mg/ml bovine serum albumin, pH 9.5. The reaction was stopped by adding 100 μl of 2N HCl followed by 100 μl of 20 mg/ml fatty acid-free bovine serum albumin (Sigma Chemical Co.). After 30 min on ice, the samples were centrifuged at 10,000 *g* for 5 min and the pellet was washed twice with 200 μl ice cold 0.1% acetic acid. The combined supernatants, which contained released [^{14}C]oleic acid, were analyzed by scintillation counting.

Egg yolk assay. PLA_2 activity was also measured as described by Habermann and Hardt (19). Briefly, fresh egg yolk (1 vol) and 0.85% NaCl (3 vol) were mixed and centrifuged at 2,000 rpm. 1 ml of supernatant was added to 98 ml of a 0.6% agarose solution in 50 mM sodium acetate/barbital buffer at 50°C (pH 7.5), followed by 1 ml of 10 mM $CaCl_2$. 15 ml of this mixture was poured into a petri dish and 3-mm wells were cut in the gel so that 5-μl test samples could be accomodated. The diameters of the clear zones were measured after overnight incubation at room temperature. In addition to the murine *i*PLA_2, we also obtained several phospholipase A_2 preparations from Sigma Chemical Co., including enzymes from bee venom, 1350 U/mg protein, bovine pancreas, 70 U/mg protein, and *Naja mocambique mocambique* 1,700 U/mg protein (acidic isozyme).

Antimicrobial testing

Bacteria. E. coli ML-35 and 35p were described previously (15). *Salmonella typhimurium* strains 14028S and 7953S were gotten from Fred Heffron of the University of Oregon Medical Center and *L. monocytogenes*, EGD was provided by Pieter Hiemstra of the University of Leiden, The Netherlands.

Methods. Gel overlay and radial diffusion assays (15) were used to identify and track the active fractions during their many cycles of purification. For radial diffusion assays, we applied 5 μl samples dissolved in 0.01% acetic acid to wells in thin (1.23 mm) agarose underlayers that contained approximately 4×10^5 bacteria per ml. After 3 h at 37°C, the underlayers were covered with an equally thin overlay of double-strength (60g/l) trypticase soy agar (BBL Microbiology Systems, Cockeysville, MD) that allowed surviving bacteria to form visible microcolonies. After an 18-h incubation at 37°C, the diameter of the clear zones were measured and, after subtracting the diameter of the well (3 mm), the difference was expressed in units (1 mm = 10 U) (15). We estimated the minimal bactericidal concentrations (eMBC) by performing linear regression analyses to determine the concentration

	5	10	15	20	25	30	35	40		IDENTITY (%)
INTESTINAL/SPLENIC										
mouse	**NIAQF**	**GEMIR**	**-LKTG**	**KRAEL**	**SYAFY**	**GCHCG**	**LGGEX**	**SPKDA**	**TD**	
mouse EF (44)	**NIAQF**	**GEMIR**	**-LKTG**	**KRAEL**	**S**					20/20 (100%)
rat (36)	**SLLEF**	**GQMIL**	**-FKTG**	**KRADV**	**SYGFY**	**GCHCG**	**VGGRG**	**SPKDA**	**TD**	28/40 (70.0%)
human (22,38)	**MLVNF**	**HRMIK**	**-LTTG**	**KEAAL**	**SYGFY**	**GCHCG**	**VGGRG**	**SPKDA**	**TD**	28/40 (70.0%)
pig (21)	**DLNNF**	**RKMIK**	**-LKTG**	**KAPVP**	**NYAFY**	**GCYCG**	**LGGEG**	**SPKDA**	**TD**	27/40 (67.5%)
REPTILIAN										
Agkistrodon halys blomhoffii (57)	**HLLQF**	**RKMIK**	**-KMTG**	**KEPVI**	**SYAFY**	**GCYCG**	**SGGRG**	**KPKDA**	**TD**	24/40 (60.0%)
PANCREATIC/GASTRIC										
human (58,59)	**AVWQF**	**RKMIK**	**CVIPG**	**SDPFL**	**EYNNY**	**GCYCG**	**LGGSG**	**TPVDE**	**LD**	18/40 (45.0%)
pig (60)	**ALNQF**	**RSMIK**	**CAIPG**	**SHPLM**	**DFNNY**	**GCYCG**	**LGGSG**	**TPVDE**	**LD**	16/40 (40.0%)

Figure 3. NH_2 terminal sequences of PLA_2. Amino acids are shown in standard single letter code, and are numbered according to reference 24. X denotes an unidentified residue. Sources: *Intestinal/splenic:* mouse *i*PLA_2 (this paper), mouse EF (43), rat (34), pig (20), human (31, 36), *Reptilian: A. blomhoffii* (49), *Pancreatic* human (50, 51), pig (52), guinea pig (53). Residues identical to mouse *i*PLA_2 are indicated in bold letters. The number and percentage of residues identical to mouse *i*PLA_2 are shown in the right column.

of peptide corresponding to 20 U of activity. This concentration, which we call the C_{20} value, was converted to μg/ml by a formula, eMBC = 0.32 (C_{20}), derived from calculations that took into account the volume of underlayer agar corresponding to a diameter of 20 U [$\frac{1}{4}\pi h(d_1^2 - d_2^2)$; where h = 1.23 mm, d_1 = 5 mm and d_2 = 3 mm], the volume of peptide placed in the well (5 μl), and the calculated C_{20}. Complete absence of micro-colonies in the clear zones that surrounded the wells was confirmed by direct microscopy under a magnification of 40. Approximately 6×10^3 micro-colonies were present in control areas equivalent in size to 20 U.

Since this assay was performed by adding very small peptide/protein samples (5 μl) to a relatively large antibiotic-free gel (10 ml), the test items underwent considerable dilution as they diffused into the underlay. Such dilution would have allowed transiently inhibited but still viable organisms to resume growth after local concentrations of the agent had fallen below its inhibitory threshhold. Consequently, although the radial diffusion assay reflects bactericidal activity accurately, it is poorly suited for measuring bacteriostatic activity, differing in this respect from conventional agar or broth dilution antibiotic assays which measure both, because the test antibiotics are uniformly dispersed throughout the medium.

Colony count assays were performed by incubating approximately 1×10^6 CFU ml^{-1} of mid-logarithmic phase bacteria with $iPLA_2$ at 37°C in 10 mM sodium phosphate buffer that contained 0.3 mg ml^{-1} of trypticase soy broth powder (BBL Microbiology Systems). At predetermined intervals, aliquots were removed, diluted and applied to the surface of nutrient agar plates with a Model C Spiral Spreader (Spiral Systems, Cincinnati, OH). Colonies were counted 24h later and converted to CFU ml^{-1} after correction for dilution and geometry

Sequences

Approximately 0.3 nmol of the purified peptide was subjected to Edman sequencing on a Porton model 2090 sequencer (Beckman Instruments, Fullerton, CA).

Results

Initial identification of phospholipase A_2. We previously reported that multiple antimicrobial components were present when Paneth cell granule extracts were tested against *L. monocytogenes* EGD in gel overlay assays (See Fig. 1 *b* in reference 6). After the initial gel permeation chromatography and RP-HPLC steps, we observed that fractions eluting at 37–38% acetonitrile consistently manifested antimicrobial activity, but did not contain cryptdins or lysozyme. After further micro-scale purification of the active component, whose apparent mass was 16 kD by SDS-PAGE (data not shown), it was electro-transferred to a PVDF membrane. Microsequencing gave the following result: NIAQFGEMIRLKTGKRAELSYAFYG. Search of the Swiss-Prot 19 computer data base using the FASTA algorithm revealed homology to phospholipase A_2 enzymes, further described below.

Purification from intestinal acetone powders. Because preparation of granules from freshly exfoliated epithelial cells was tedious and ultimately provided only a few micrograms of purified *i*PLA_2, we turned to commercial mouse intestinal acetone powders to obtain material for its larger scale purification. After acid extraction, purification was accomplished with a five-step procedure consisting of: size selection by ultrafiltration, RP-HPLC, cation-exchange HPLC, preparative AU-PAGE and final RP-HPLC (Fig. 1). RP-HPLC followed by cation exchange-HPLC of the YM-5 retentate yielded a substantially purified preparation with one major and 2 minor components noted on AU-PAGE. Phospholipase A_2, the major and most cationic component, was separated from the minor contaminants by preparative continuous AU-PAGE (17). Unless otherwise stated, the final *i*PLA_2 preparations used for the experiments described herein appeared homogeneous by RP-HPLC, AU-PAGE, and SDS-PAGE analysis.

Enzyme activity. In the ^{14}C-labeled *E. coli* assay (18), purified murine intestinal phospholipase A_2 was about 10-fold more active than bovine pancreatic phospholipase A_2 (Fig. 2), and slightly more active than a phospholipase A_2 derived from Cobra venom (*Naja mocambique mocambique*). As expected (20, 21), murine *i*PLA_2 was inactive in the classical egg yolk assay at 10 μg/ml, the highest concentration we tested (data not shown).

Sequence analysis. The partial, NH_2-terminal amino acid sequence of purified murine intestinal phospholipase A_2 is shown in Fig. 3, which also compares it with other PLA_2 enzymes and to murine intestinal "Enhancing Factor" (see Discussion). The homologies and especially the absence of the cysteine residue universally present at position 11 in Type 1 PLA_2 enzymes, identifies the murine protein as a Type 2 PLA_2.

Antimicrobial activity. Purified murine *i*PLA_2 killed *E. coli* ML-35p and *L. monocytogenes EGD,* as shown by the radial diffusion assays illustrated in Fig. 4, which were performed in the absence of additional calcium. On a weight basis *i*PLA_2 ($M_r \approx 16$ kD) was 3–4-fold less potent than rabbit defensin NP-1 ($M_r \approx 4$ kD), but on a molar basis their efficacy was similar. Although *i*PLA_2 killed both mouse-virulent wild-type *S. typhimurium* strain 14028S and its avirulent *phoP* mutant, strain 7953S (Fig. 5 and Table I), the latter was approximately 3.5×

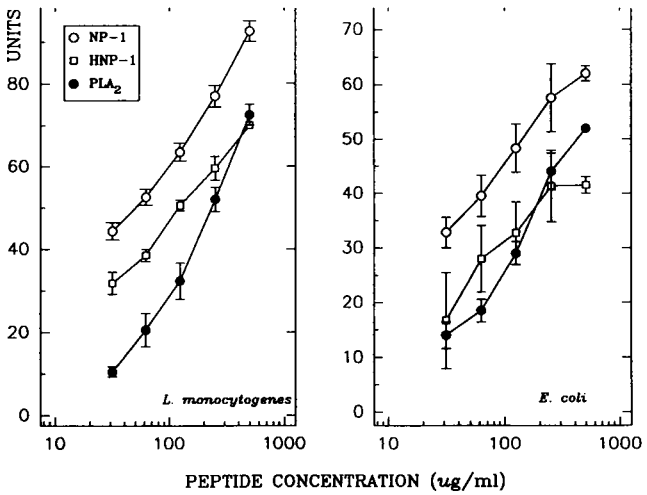

Figure 4. Activity against *L. monocytogenes* and *E. coli.* The bactericidal activities of murine *i*PLA_2, rabbit defensin NP-1 and human defensin HNP-1 to kill *L. monocytogenes,* strain EGD and *E. coli,* strain ML-35p were compared in radial diffusion assays. Data show mean±SEM values from four independent experiments.

more sensitive ($P < 0.001$). The differential susceptibility of these isogenic *S. typhimurium* strains to *i*PLA_2 was confirmed by colony counting experiments (Fig. 6), as was the bactericidal effect of *i*PLA_2 on *E. coli* (data not shown). Table I also shows results obtained when these *Salmonella* strains were challenged with human defensin HNP-1 and a rabbit defensin (NP-2) which is identical in 32 of its 33 residues to NP-1.

The ability of *i*PLA_2 to kill *L. monocytogenes* shown in Figs. 4 and 7 was confirmed by colony count experiments, which showed that concentrations of 12.5 μg/ml or greater sterilized the culture within 60 min (Fig. 8 *a*) and that the listericidal effect occurred quite rapidly (Fig. 8 *b*). Fig. 7 also shows that *i*PLA_2-mediated listericidal activity was highly calcium-dependent. Addition of calcium considerably enhanced the listericidal activity of *i*PLA_2, whereas addition of EGTA, a calcium chelator, greatly diminished it. In contrast, magnesium addition did not enhance listericidal activity (data not shown). Fig. 7 also shows that calcium concentrations had little effect

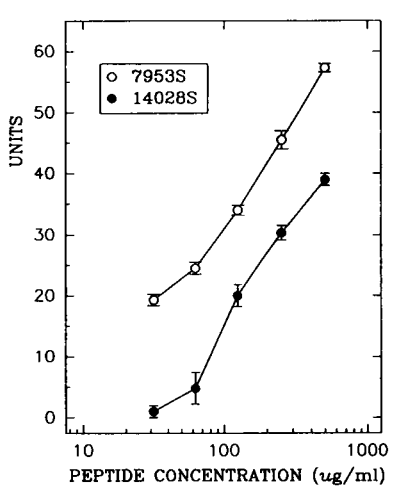

Figure 5. Activity against *S. typhimurium.* Mouse iPLA2 was tested against isogenic *S. typhimurium* strains. Strain 14028S (wild-type) is mouse-virulent and Strain 7953S, its isogenic *phoP* mutant, is avirulent (54, 55).

Table I. Relative Potency of Defensins and iPLA$_2$

Test organism	Strain	*i*PLA2	NP1/NP2*	HNP-1
		μg/ml	μg/ml	μg/ml
L. monocytogenes	EGD	18.8±2.4	2.6±0.6	4.9±0.9
E. coli	ML-35p	18.9±2.6	4.1±0.4	12.9±6.1
S. typhimurium	14028S	43.9±4.5	14.2±1.9*	62.7±7.1
S. typhimurium	7953S	12.7±0.5	5.6±1.0*	4.1±0.5

Estimated minimal bactericidal concentrations were estimated from radial diffusion assays and are shown as means ± SEM. In some experiments (*) rabbit defensin NP-2 was used instead of NP-1. 4–6 individual experiments were performed in the absence of added calcium with each bacterium/peptide combination. Rabbit and human defensins were purified from leukocytes as previously described (61).

on the listericidal activity of defensins. Consonant with these indications that enzymatic activity is required for *i*PLA_2-mediated bactericidal activity, we also found that reduced and alkylated *i*PLA_2, which was enzymatically inactive, failed to kill *E. coli* ML-35 or *L. monocytogenes* (data not shown).

Although phospholipase A_2 activity appeared to be necessary for *i*PLA_2-mediated bactericidal activity, enzymatic activity alone was not sufficient, since bee venom PLA_2 was much less effective than *i*PLA_2 against *L. monocytogenes,* (Fig. 9) even though it was much more potent than *i*PLA_2 in releasing [^{14}C]-oleic acid from radiolabeled autoclaved *E. coli* in our enzymatic assays (Fig. 2).

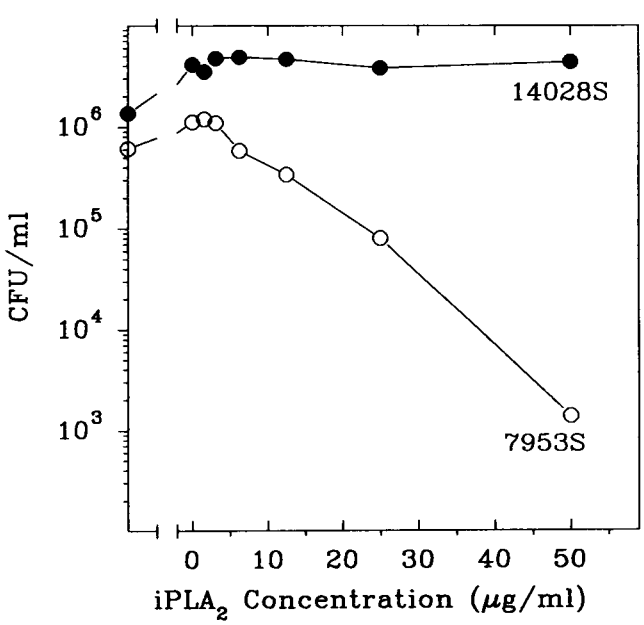

Figure 6. Activity against *S. typhimurium.* Mid-logarithmic phase *S. typhimurium* 14028S and 7953S were incubated for 60 min at 37°C with the indicated concentrations of *i*PLA_2. The incubations were performed in a dilute trypticase soy broth medium containing 10 mM sodium phosphate buffer, pH 7.4, and 0.30 mg/ml of trypticase soy broth powder. The input concentrations are shown on the ordinate.

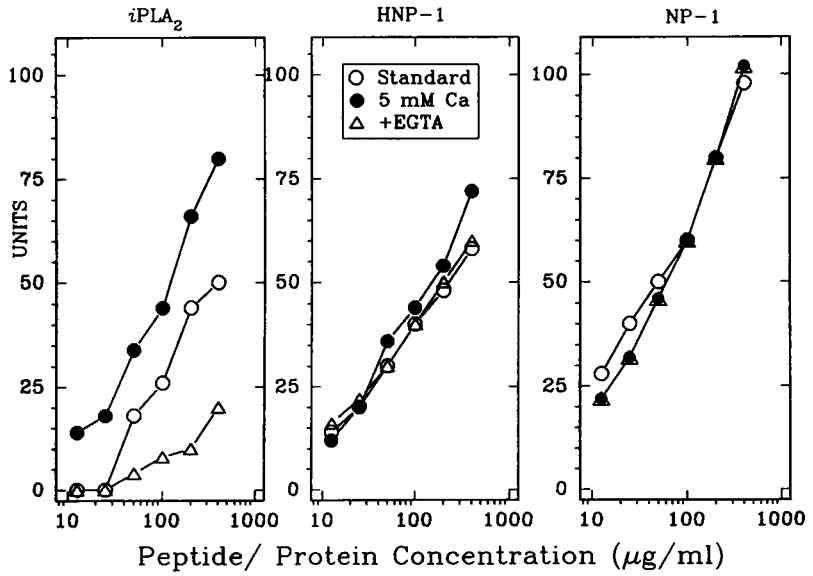

Figure 7. Activity against *L. monocytogenes*. Mouse iPLA2 was tested against *L. monocytogenes* in underlayers that contained 10 mM Hepes without added calcium, 10 mM HEPES + 5 mM calcium or 10 mM Hepes + 1.25 mM EGTA. Also tested under these conditions were human defensin HNP-1 and rabbit defensin NP-1. In this experiment, which was performed with the > 95% pure iPLA$_2$ preparation, the underlay agarose gel was buffered with 10 mM Hepes, pH 7.4

Discussion

Type II PLA$_2$, which is present in the granules of rat and human intestinal Paneth cells (13, 14), hydrolyses phosphatidylglycerol, a major bacterial phospholipid, several hundred times more rapidly than phosphatidylcholine, the principal phospholipid of egg yolk (20–22). In a recent report characterizing human ileal PLA$_2$, Minami et al. stated that the physiological role of iPLA$_2$ was unknown, but noted its production by Paneth cells which contained other molecules (lysozyme, IgA, and defensin) that might control the local bacterial milieu (22). The present report provides the first demonstration that iPLA$_2$ is an effective microbicide, whose direct antibacterial potency against *E. coli, S. typhimurium,* and *L. monocytogenes* approximates that of defensins on a molar basis, even when relatively low concentrations of calcium are present.

All PLA$_2$ enzymes (E.C. 3.1.1.4) hydrolyze the sn-2 fatty acyl moiety from phospholipids, releasing equimolar amounts of free fatty acids and lysophospholipids. Although these enzymes are often described according to their tissues of origin, as "pancreatic," "splenic," or "intestinal" PLA$_2$, it is more useful to classify them according to their structural features and primary sequence. Type I PLA$_2$ is exemplified by mammalian pancreatic enzymes and homologs found in "old world" elapid snake venoms, whereas the Type II enzymes include intestinal and splenic PLA$_2$s and venom constituents of "new world" viperid and crotalid snakes (23, 24). The half-cystine residues found at positions 11 and 77 in Type I PLA$_2$ are transposed to positions 50 and 133 in Type II PLA$_2$s, numbered according to Renetseder et al. (25). Type II enzymes also contain a short C-terminal extension, absent in Type I PLA$_2$s, that ends with a half-cystine residue (23). Although we sequenced only 40 residues of the murine intestinal PLA$_2$, the absence of a half-cystine residue at position 11, and the protein's size (~ 16 kD), overall homology to the human and rat intestinal PLA$_2$ (Fig. 3), and enzymatic activity make its identification and classification secure. The primary sequence of murine intestinal PLA2 includes lipophilic residues (Ile^2, Phe^5, Ile^9) at characteristic positions of the α-helical amino terminal segment which also contains several basic residues, and an intact glycine-rich domain (Tyr^{25}-Gly-Cys-X-Cys-Gly-X-Gly-Gly-X-X-X-Pro) that can bind calcium. (26)

Phospholipase A$_2$ enzymes are found in all cell membranes, and in the secretory granules (and secretions) of certain cells. The intracellular forms play key roles in phospholipid metabolism (27), membrane remodeling and repair (28), biosynthesis of eicosanoid lipid mediators (29), and G-protein–regulated receptor-mediated signal transduction (30). Extracellular phospholipase A$_2$ is present in mammalian pancreatic secretions, snake and arthropod venoms (31), and Paneth cell secretions (see Abstract) and is released by thrombin-stimulated platelets (32). Elevated blood or inflammatory fluid levels of Type 2 PLA$_2$ are found in association with rheumatoid arthritis, septic shock, ulcerative colitis and Crohn's disease, and may contribute prominently to the pathophysiology of these disorders (31, 33, 34).

The enzyme we call "intestinal" PLA$_2$ in this report has a considerably wider tissue distribution than the Paneth cell. The human "intestinal" and "splenic" Type 2 -PLA$_2$ are identical (22, 35), as are their counterparts in rats (36). Moreover, these "intestinal/splenic" Type 2 PLA$_2$s are apparently identical to the enzymes recovered from platelets (32, 37), various inflammatory exudates (26, 35, 38–40) and placenta (41). Consequently, Type 2 PLA$_2$ could also mediate non-oxidative antibacterial activity in non-intestinal sites. Although this report deals only with the direct antibacterial effects of intestinal PLA$_2$ acting in isolation, the enzyme may act in concert with other antimicrobial host molecules, as has been shown with respect to degradation of phospholipids in BPI-treated *E. coli* by Elsbach and associates (42, 43).

Recently, Mulherkar et al. (44, 45) identified the 20 NH$_2$-terminal residues of "EF" (enhancing factor), a mouse small intestinal protein that promoted fibroblast growth in the presence of EGF and increased EGF binding to receptor-positive fibroblasts and epidermoid carcinoma cells, as well as to EGF-receptorless NR-6 cells. They reported that EF had phospholipase A$_2$ activity (43). As indicated in Fig. 3, the 20–amino terminal residues of EF are identical to those of the intestinal

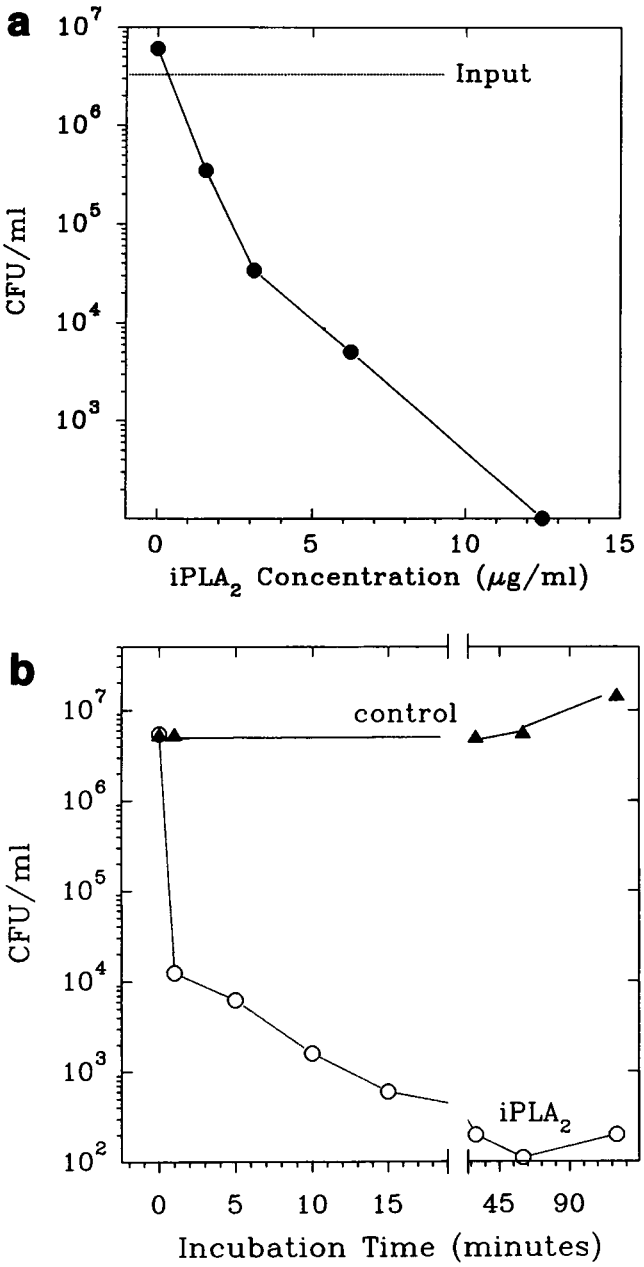

Figure 8. Activity against *L. monocytogenes*. (*a*). Approximately 3 × 10^6 CFU/ml of mid logarithmic phase bacteria were incubated for 60 min at 37°C with the indicated concentrations of $iPLA_2$ in the medium described in the legend for Fig. 6. (*b*) Approximately 4 × 10^6 CFU/ml of mid logarithmic phase bacteria were incubated for the indicated times with 12.5 μg of $iPLA_2$ or in its absence (control).

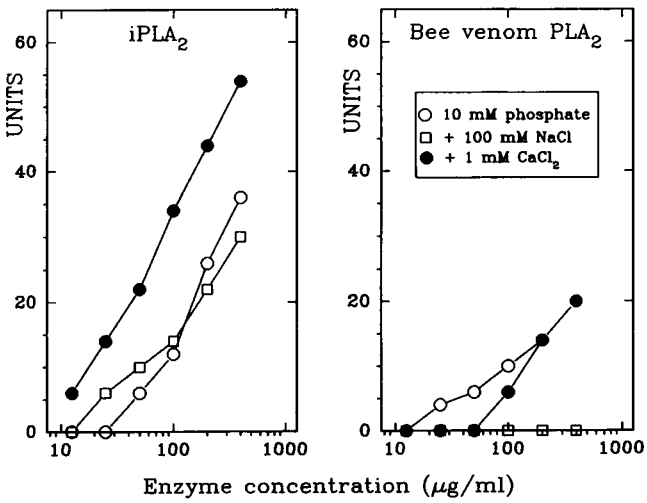

Figure 9. Comparison of $iPLA_2$ and bee venom PLA_2. The murine and bee venom enzymes were tested for bactericidal activity against *L. monocytogenes* in agarose underlay gels buffered with 10 mM sodium phosphate, pH 7.4. Some underlayes also contained 1 mM $CaCl_2$ or 100 mM NaCl, as indicated in the inset legend.

phospholipase A_2 we purified, virtually assuring that they are identical. Thus, Paneth cell-derived $iPLA_2$ may not only help to protect intestinal crypts from bacterial incursions, it may also foster the vital processes of epithelial cell renewal that occur within the crypts.

How does Type 2 PLA_2 kill bacteria? Although we cannot answer the question definitively, a few comments are appropriate. Due to their high ratio of basic (R + K + H) to acidic (D + E) amino acids, Type 2 intestinal/splenic A_2 phospholipases are basic proteins with isoelectric points that exceed 10.5 (32). Their strongly cationic nature favors electrostatic interactions with the (poly)anionic constituents typically found in bacterial cell walls and membranes, and could promote the occurrence of locally high protein concentrations at these vital sites. A correlation between regional (amino terminal) cationicity and the ability to bind to BPI-treated *E. coli* has been shown for various venom enzymes (46, 47). Indeed, we have noted that the basic (pI 9.6) isoform of PLA2 from the cobra *Naja mocambique mocambique* is powerfully microbicidal for *S. typhimurium*, whereas an acidic isoform of this cobra venom enzyme is nonbactericidal (unpublished data). Although ionic interactions between basic PLA_2 molecules and the bacterial surface should be relatively nonspecific, this process could be further augmented by the unusual affinity of intestinal PLA_2 for bacterial phospholipids relative to other phospholipase substrates (Fig. 2, references 20–22).

Although our data strongly suggest that enzymatic activity is required for the antibacterial effects of Type 2 $iPLA_2$, they do not exclude the possibility that $iPLA_2$ also contains domain(s) that could manifest enzyme-independent antibacterial activity, as reported for neutrophil-derived cathepsin G (48) and azurocidin/CAP37 (49). However, $iPLA_2$ is a complexly folded molecule that is stabilized by six intramolecular cystine disulfides and when we reduced murine $iPLA_2$ and alkylated it to prevent spontaneous renaturation, the linearized molecule lost > 99.9% of its enzymatic activity and > 99% of its antimicrobial activity. Although this result merely indicates that intact secondary structure is essential for both enzymatic and bactericidal activity, it also mitigates against the likelihood that the molecule can exerts appreciable enzyme-independent antimicrobial activity. Although further delineation of the precise bactericidal mechanism of $iPLA_2$ is needed, the use of Paneth cell secretions (50) or tissues other than mouse intestine (e.g., human or animal spleen, platelets or leukocytes) are more likely

to provide the amounts of Type 2 iPLA$_2$ necessary for such studies.

Our finding that the phoP mutant, *S. typhimurium* 7953S, is much more susceptible to iPLA$_2$ than its isogenic parent *S. typhimurium* 14028S is especially noteworthy. PhoP is the regulatory component of a bacterial transcriptional activator/sensory kinase regulon which modulates the production of a cohort of proteins, some of which are virulence-determining (51). PhoP mutants show an impaired ability to survive in murine macrophages (52, 53), as well as greater susceptibility to low pH (54), and to certain antimicrobial peptides (55, 56). *S. typhimurium* normally infects its hosts via the gastrointestinal tract, most likely penetrating the intestinal epithelium in Peyer's patches in the distal ileum and then surviving its initial encounters with intestinal macrophages. During this journey, *S. typhimurium* is likely to encounter type II PLA$_2$ molecules at least twice, first in Paneth cell secretions and next in the lysosomal apparatus of the macrophage. Consequently, phoP-regulated resistance to type II PLA$_2$ could ontribute significantly to virulence in this group of enteric pathogens.

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