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Melittin Binds to Secretory Phospholipase A_2 and Inhibits Its Enzymatic Activity

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Synthetic melittin inhibited the enzymatic activity of secretory phospholipase A_2 (PLA_2) from various sources, including bee and snake venoms, bovine pancreas, and synovial fluid from rheumatoid arthritis patients, irrespective of substrate (e.g., [^{14}C]-phosphatidylcholine or phosphatidylethanolamine vesicles and [^{3}H]-oleic acid-labeled *E.coli*). A Lineweaver-Burk analysis showed that melittin was a noncompetitive inhibitor of bee venom PLA_2, causing a change in V_{max} from 200 to 50 units/min/mg of protein. The K_m remained unchanged (0.75 nmole). Melittin inhibited approximately 50% of purified bee venom PLA_2 activity in a 30:1 molar ratio (melittin:enzyme). Because the enzyme kinetics indicated a PLA_2-melittin interaction, a melittin-sepharose affinity column was used to purify a PLA_2 from human serum. Further, an enzyme-linked assay was developed to quantitate PLA_2 activity in biological fluids using avidin-peroxidase and ELISA plates coated with biotinylated melittin. These observations may have potential therapeutic significance, as well as provide a convenient basis for the isolation and quantitation of PLA_2.

Key Words: **phospholipase A_2; melittin; phospholipid; fatty acid; bee venom; snake venom; bovine pancreas; rheumatoid arthritis; synovial fluid; enzyme kinetics; inflammation.**

Melittin is a 26-amino acid peptide that is a major component of bee venom, comprising 50% of its dry wt (1). It is widely known as a potent enhancer of PLA_2 activity, although results may vary depending upon whether the liposome substrate was sonicated (2) or unsonicated (3). Activation of endogenous PLA_2 in intact cells by melittin has been reported (4-6), and melittin has been used as a probe for stimulating endogenous PLA_2 activity (7). In some cases, enhancement of PLA_2 activity by purified melittin could have been due to contamination with heat-stable PLA_2 (8); however, some investigators have not detected PLA_2 contamination in melittin used in their studies (6,9). Bee venom PLA_2 contamination of commercial, purified melittin has been reported to be the presumed activator of 75% of tissue PLA_2 activity (10). In contrast, recent studies showed enhancement of PLA_2 activity in a unilamellar system with purified melittin (11), as well as in PC12 cells with synthetic melittin (12). Because of this discrepancy in the results of various investigators on the effect of melittin on PLA_2 activity, the present study was undertaken to examine the *in vitro* effect of synthetic melittin, free of any contaminating PLA_2 enzyme, on the enzymatic activity of secretory PLA_2 from bee venom, snake venom, bovine pancreas, and synovial fluid from rheumatoid arthritis patients.

MATERIALS AND METHODS

Reagents. Synthetic melittin and biotinylated synthetic melittin were prepared by the Synthetic Antigen Laboratory at M.D. Anderson Cancer Center, Houston TX. Cold arachidonic acid, streptavidin-peroxidase, ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), purified PLA_2 from bee venom and snake venom, as well as from bovine pancreas were obtained from Sigma Chemical Company, St. Louis MO. Synovial fluid was collected from patients at John Sealy Hospital, Galveston, Texas. Radiolabeled [^{3}H-(N 9,10)]-oleic acid was obtained from American Radiolabeled Chemicals Inc., St. Louis, MO, and radiolabeled phosphatidylcholine L-α-1-palmitoyl-2-arachidonyl [arachidonyl-1-^{14}C] and phosphatidylethanolamine L-α-1-palmitoyl-2-arachidonyl [arachidonyl-1-^{14}C] were purchased from DuPont-NEN, Boston, MA. Human serum was provided by Gemini BioProducts Inc., Calabasas, CA. Glass silica gel plates (Whatman LK6DF) were purchased from Fisher Scientific, Houston TX, while epoxy-activated sepharose 6B was from Pharmacia Biotech, Uppsala, Sweden. All other chemicals were of ultrapure grade.

PLA_2 assay using phospholipid vesicles. The procedure of Hildebrandt and Albenesi (13) was followed with some modifications. Briefly, radiolabeled phosphatidylcholine or phosphatidylethanolamine containing [^{14}C]-arachidonic acid in the *sn*-2 position of the phospholipid was dried under N_2, and lipid vesicles were prepared in 0.05% Triton X-100. The reaction mixture contained 10 μl of phospholipid substrate, 10 μl of 5× assay buffer (500 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5.0% fatty acid-free BSA, and 5 mM $CaCl_2$), 10 μl of PLA_2 enzyme preparation, and sufficient H_2O to adjust the total volume to 50 μl. The reaction mixture was incubated for 30 min at

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37°C in a waterbath and the reaction terminated by the addition of 50 μl of chloroform and methanol (1:3), containing 200 μg/ml of unlabeled arachidonic acid. Lipid was extracted by adding an additional 50 μl of chloroform and 50 μl of 4 M KCl, after which the mixture was vortexed and centrifuged in a microfuge at 13,000 rpm for 10 min. A 25-μl aliquot of the lower phase was spotted onto glass silica gel plates and placed in a thin layer chromatography chamber with a solvent system containing petroleum ether, diethyl ether, and acetic acid (75:25:1) for 30 min. The lipid spots were made visible by placing the silica gel plates in a chamber containing iodine vapor. The silica gel area corresponding to arachidonic acid was scraped from the plates and transferred to scintillation vials containing 10 ml of liquid scintillation cocktail (ScintiVerse, Fisher Scientific, Houston, TX). The amount of radionuclide corresponding to [^{14}C]-arachidonic acid in each vial was determined in a Beckman liquid scintillation counter.

PLA_2 assay with [^{3}H]-oleic acid-labeled E. coli. A modification of the procedure described by Elsbach and Weiss (14) provided an additional method for measuring PLA_2 activity. *E. coli* strain SJ198, kindly provided by Dr. Suzanne Jackowski (St. Jude Children's Research Hospital, Memphis, TN) was grown in 10 ml of M-9 minimal medium plus 50 μl glycerol, 100 μl vitamin mix, 40 μl methionine (25 mg/ml), 50 μl [^{3}H]-oleic acid (0.1 μCi/μl), and 100 μl casamino acids (100 mg/ml). After incubation at 37°C overnight, the culture was centrifuged and washed in PBS (3×), autoclaved for 15 min, and washed again (3×) before storing at −70°C. The reaction mixture contained 10 μl of 5× assay buffer, 10 μl of the PLA_2 enzyme preparation, 10 μl of [^{3}H]-oleic acid-labeled *E. coli* cells, and enough H_2O to adjust the total volume to 50 μl. The reaction mixture was incubated for 15 min at 37°C in a waterbath. To terminate the reaction, the tubes were transferred immediately to an ice bath, and an equal volume of either 1 M HCl or 4 volumes of cold PBS was added. After centrifugation in a microfuge at 4°C for 10 min, an aliquot equal to 25% of the volume of the supernatant was used to measure the amount of [^{3}H]-labeled oleic acid released from the *E. coli* cells by PLA_2 using liquid scintillation counting.

Binding affinity of biotinylated melittin to PLA_2. Each well of the ELISA plates was coated with 100 μl of biotinylated synthetic melittin (1 μM), dissolved in sodium bicarbonate buffer (0.1 M, pH 8.2), overnight at room temperature. After washing 3× with PBS, containing 0.05% Tween 20, and blocking with 10% BSA, two-fold serial dilutions of synovial fluid in PBS containing 1% BSA were added and the reaction mixture was incubated at 37°C for 2 hr. After washing as above, 100 μl of streptavidin-peroxidase (2.5 mg/ml) diluted in PBS containing 1% BSA was added, and the reaction was incubated for another 2 hr. After washing (5×), color was developed with 100 μl of 2,2′-Azino-bis ABTS substrate solution (0.3 mg/ml), prepared in 0.1 M sodium citrate buffer, pH 4.4, containing freshly added 30% H_2O_2 (1 μl/ml). The optical density of the reaction product was monitored at 405 nm in an ELISA plate reader (Molecular Devices, Menlo Park, CA).

Conjugation of melittin to epoxy-activated sepharose. Synthetic melittin was conjugated to epoxy-activated sepharose 6B. Briefly, 10 g of epoxy-activated sepharose 6B was washed with 2.0 L of H_2O and 1.5 L of sodium carbonate buffer (0.1 M, pH 10.8). Synthetic melittin (5 mg) was dissolved in distilled H_2O (2 ml) and added to the gel slurry (5 ml). The final volume of the slurry was increased to 30 ml with sodium carbonate buffer (0.1 M, pH 10.8) and allowed to shake overnight at room temperature. Unreacted sites were blocked with ethanolamine (1.0 M, pH 8.0) by incubating at 43°C for 3 hr and then transferring to a shaker at room temperature overnight. The gel slurry was washed 3 × with alternating cycles of Tris-HCl buffer (100 mM containing 500 mM NaCl, pH 8.0) and sodium acetate buffer (100 mM containing 500 mM NaCl, pH 4.0) followed by washing with sodium phosphate buffer (10 mM containing 150 mM NaCl, pH 7.0) before packing into a glass column.

Isolation of a PLA_2 from human serum. Ten milliliter of heat-inactivated human serum (56°C, 30 min) was diluted 1:10 with PBS and loaded onto a melittin-sepharose column pre-equilibrated with PBS. The column was washed with 500 ml PBS overnight and the bound PLA_2 was eluted with 0.5 M glycine-HCl buffer, pH 2.8. Two-ml fractions were collected, neutralized to pH 7.2 with Tris-HCl buffer, and analyzed for PLA_2 activity using [^{3}H] oleic acid-labeled-*E. coli* cells as substrate. Eluted fractions were concentrated and dialyzed in Centricon 10 filter units (Amicon, Inc., Beverly, MA), and the pass through was discarded. The retentate was again passed through the Centricon 30, and the volume was reduced to about one-fifth that of the original volume. Both the retentate and pass through volumes were assayed for PLA_2 activity in the presence and absence of melittin.

Protein quantitation. Protein content was determined by the method of Bradford (1976), using BSA as a standard (15).

Statistical analysis. Arithmatic mean (±) and standard deviation (SD) were derived from triplicate and/or duplicate values. The data were evaluated with an unpaired Student's t-test (one-tailed), and values ($p<0,05$), were considered to be significantly different from those of controls.

RESULTS

Inhibition of PLA_2 activity by melittin in cell-free enzyme assays. Synthetic melittin inhibited the enzymatic activity of secretory PLA_2 from various sources, including bee venom, snake venom, bovine pancreas, and synovial fluid from rheumatoid arthritis patients. Two separate assays were used to estimate PLA_2 activity, including a thin-layer chromatography (TLC) method with either [^{14}C]-arachidonic acid-labeled phosphatidylcholine or [^{14}C]-arachidonic acid-labeled phosphatidylethanolamine substrate, as well as a [^{3}H]-oleic acid-labeled *E. coli* release method. The results from these assays are summarized in Table 1 (A-C). Melittin (1 μg/ml) caused a significant inhibition in the PLA_2 activity of bee venom (84%), snake venom (93%), bovine pancreas (64%) and synovial fluid (96%), when [^{14}C]-arachidonic acid-labeled phosphatidylcholine substrate was used (Section A, Table 1) ($p \leqslant 0.01$-0.001). However, when phosphatidylethanolamine substrate was used (Section B), a significant decrease in the PLA_2 activity was observed for bee venom PLA_2 (46%), snake venom (93%), synovial fluid (88%), and bovine pancreas PLA_2 (61%) ($p \leqslant 0.05$-0.01). Finally, using [^{3}H]-oleic acid-labeled *E. coli* as substrate (Section C) and cold PBS to terminate the reaction, melittin (10 μg/ml) caused maximal inhibition (96%) of bovine pancreatic PLA_2 activity ($p \leqslant 0.001$). This was followed by PLA_2 from synovial fluid (93%, $p \leqslant 0.01$), snake venom (57%, $p \leqslant 0.01$), and bee venom (19%, $p \leqslant 0.05$). In contrast, when HCl was used to stop the reaction of PLA_2 in the *E. coli* assay (Table 1 - section D), a statistically significant enhancement in the PLA_2 activity of bee venom (68%, $p \leqslant 0.001$) and synovial fluid (33%, $p \leqslant 0.01$) was observed.

To determine whether acidification of the *E. coli* cells enhanced the release of [^{3}H]-oleic acid, we incubated different concentrations of melittin with the [^{3}H]-oleic acid-labeled *E. coli* substrate at 0°C and 37°C for 15

TABLE 1

Effect of Melittin on the Activity of Secretory PLA_2 from Bee Venom, Snake Venom, Bovine Pancreas, and Synovial Fluid from Rheumatoid Arthritis Patients, Using Vescicles Labeled with [^{14}C]-Phosphatidylcholine (A) or Phosphatidylethanolamine (B) and [^{3}H]-Oleic Acid-Labeled *E. coli* as substrate (C&D)

	Phospholipase A_2 activity			
Enzyme source	Bee venom	Snake venom	Bovine pancreas	Synovial fluid
A. [^{14}C]-Phosphatidylcholine as substrate				
No peptide	5.63 ± 0.25	18.81 ± 0.26	$9.9 \pm 0.3 \times 10^{-4}$	$3.61 \pm 0.18 \times 10^{-3}$
Melittin (1 μg/ml)	0.90 ± 0.07***	1.41 ± 0.84***	$3.6 \pm 0.9 \times 10^{-4}$ **	$0.11 \pm 0.6 \times 10^{-3}$ **
	84%↓	93%↓	64%↓	96%↓
B. [^{14}C]-Phosphatidylethanolamine as substrate				
No peptide	36.17 ± 1.24	0.71 ± 0.08	$5.21 \pm 1.70 \times 10^{-2}$	$6.93 \pm 0.25 \times 10^{-3}$
Melittin (1 μg/ml)	19.66 ± 2.08**	0.05 ± 0.03**	$2.04 \pm 0.92 \times 10^{-2}$ *	$0.8 \pm 0.3 \times 10^{-3}$ **
	46%↓	93%↓	61%↓	88%↓
C. [^{3}H]-oleic acid-labeled *E. coli* as substrate/PBS				
No peptide	989 ± 28.31	143.47 ± 7.8	1.61 ± 0.01	73.7 ± 3.9
Melittin (10 μg/ml)	801 ± 55.0*	61.5 ± 2.75**	0.06 ± 0.03***	5.5 ± 0.4**
	19%↓	57%↓	96%↓	92%↓
D. [^{3}H]-oleic acid-labeled *E. coli* as substrate/HCl				
No peptide	779.0 ± 40.0	46.01 ± 18.8	1.47 ± 0.27	29.09 ± 1.15
Melittin (10 μg/ml)	1315.0 ± 48.2***	59.13 ± 13.97	1.51 ± 0.17	43.6 ± 3.19**
	68%↑	28%↑	3%↑	33%↑

Note. PLA_2 activity was expressed as μmole/min/mg of enzyme in case of purified PLA_2 enzyme, while that in synovial fluid was expressed as mmole/min/ml. In section C, the reaction was stopped by diluting the mixture with four volumes of cold PBS, while in section D the reaction was stopped by adding an equal volume of 1N HCl. Each value represents a mean ±SD. Significant differences from control values are indicated by *$p \leqslant 0.05$; **$pp \leqslant .01$; ***$p \leqslant 0.001$. Arrows (↑) and (↓) indicate increase and decrease in PLA_2 activity.

min in the absence of any PLA_2 enzyme, and the reaction was terminated either by the addition of 1 N HCl or by dilution with cold PBS. The results shown in Figure 1 indicate that following incubation at 0°C and the use of cold PBS to stop the reaction, melittin had no apparent effect on the *E. coli* substrate. Further, no release of [^{3}H]-oleic acid was observed when the concentration of melittin was increased. In contrast, a dose-dependent increase in the release of [^{3}H]-oleic acid was observed when HCl was used to stop the reaction, irrespective of the incubation temperature (i.e., 37°C or 0°C). Melittin at a concentration of 8 μg/ml, caused maximal release of [^{3}H]-oleic acid at 0°C with HCl when the reaction was stopped immediately without further incubation.

Enzyme kinetics of bee venom PLA_2 inhibition by melittin. By adding various substrate concentrations (0.125, 0.14, 0.166, 0.20, 0.25, 0.33, 0.5 and 1.0 ng/ml) to the reaction mixture, the effect on bee venom PLA_2 activity was evaluated in the presence and absence of melittin (1 μg/ml). The results were plotted as 1/[S] vs 1/[V] (Fig. 2). Melittin decreased the V_{max} from 200 μmol/min/mg of enzyme to 50 μmol/min/mg of enzyme, while the K_m value remained unaltered in both the cases (0.75 nmole).

EC_{50} of melittin on bee venom PLA_2. When we used a single concentration of purified bee venom PLA_2 (1 nM), we determined that melittin interacted with bee venom PLA_2 approximately in a 30:1 molar ratio, causing about 80% inhibition of PLA_2 enzyme activity (Fig. 3). The amount of melittin required to cause approximately 50% inhibition of PLA_2 activity *in vitro* was very low ($ED_{50}=2\times10^{-8}$M).

In vitro binding affinity of biotinylated melittin to PLA_2. The results in Fig. 4 indicate that PLA_2 in synovial fluid interfered with strepavidin-peroxidase binding to the biotinylated melittin on the plates, and the binding inhibition was linear with 2-fold dilutions of synovial fluid.

Purification of PLA_2 from human serum. A melittin-sepharose column was loaded with human serum (10 ml), washed with PBS until the eluant was negative for PLA_2 activity, and the bound PLA_2 was then eluted with glycine buffer (0.5 M, pH 2.8). PLA_2 activity was detected in fractions (2-5) with maximum activity in fraction 3. These fractions were concentrated with a Centricon 10 concentrator. PLA_2 activity and the protein content of the Centricon 10 concentrate, as well as that of human serum was determined. The specific activity of the PLA_2 in the Centricon 10 concentrated fraction and the serum were calculated. The results, summarized in Table 2A, show a 1,521-fold purification of PLA_2. A portion of Centricon 10 concentrate was

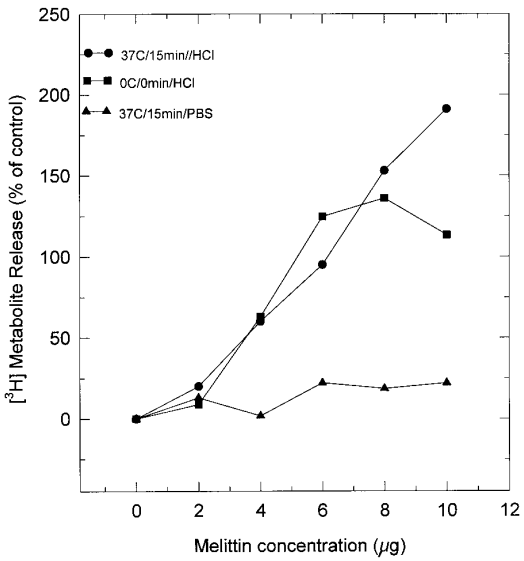

FIG. 1. Effect of melittin on the release of [^{3}H]-oleic acid from [^{3}H]-oleic acid-labeled *E. coli* cells. Various concentrations of melittin (2-10 μg/ml) were added to [^{3}H]-oleic acid-labeled *E. coli* substrate in the presence and absence of PLA_2, as outlined in the procedure for the PLA_2 assay. Tubes were incubated at 0 or 37°C, and the reaction was stopped by addition of an equal volume of 1 N HCl or 4 volumes of cold PBS, before centrifuging in a microcentrifuge at 4°C. Liquid scintillation counting was used to assess the radioactivity of the supernatants of reactions incubated at 37°C for 15 min stopped by HCl (●), 0°C for 15 min stopped by PBS (▲), and 0°C for 0 min stopped by HCl (■).

further concentrated in Centricon 30 concentrator. PLA_2 activity was determined with or without melittin, and the results are summarized in Table 2B. It appears that melittin causes about a fourfold reduction in the PLA_2 activity in the Centricon 10, as well as Centricon 30 retentate samples.

DISCUSSION

Melittin inhibited the enzyme activity of secretory PLA_2 from bee venom, snake venom, bovine pancreas, and synovial fluid from rheumatoid arthritis patients, following a thin layer chromatography (TLC) method of PLA_2 assay. In contrast, when we used [^{3}H]-oleic acid *E. coli* substrate, addition of melittin enhanced the release of label when the reaction was terminated by lowering the pH of the reaction mixture with HCl, as described in the original protocol (14). However, when the reaction was stopped by dilution with cold PBS, the inhibitory effect of melittin on PLA_2 activity was similar to that observed following TLC (Table 1). The enhancement in PLA_2 activity by melittin, on terminating reaction with HCl, could be attributed to the nonenzymatic release of [^{3}H]-oleic acid label from the *E. coli* cells at low pH. In a control experiment in which no PLA_2 was added, a dose-dependent increase in the release of [^{3}H]-oleic acid was observed with melittin, even at 0°C after addition of 1 N HCl to the reaction mixture. This dose-dependent release of [^{3}H]-oleic acid was maximum at 8 μg/ml and decreased thereafter (Fig. 1). We have considered the possibility that at low pH, an excess of melittin tends to aggregate, possibly by forming multimers. Melittin has been reported to be monomeric in water, but aggregates to form tetramers (16).

The Lineweaver-Burk plot (Fig.2) showed that the reaction catalyzed by bee venom PLA_2 was inhibited by melittin *via* a noncompetitive inhibition reaction, in which the inhibitor binds to a site on the enzyme other than the catalytic site (17, 18). Melittin interacts with bee venom PLA_2 (Sigma) in an approximate 30:1 molar ratio, causing about 80% inhibition of PLA_2 enzyme activity (Fig. 3). The amount of melittin required to cause an approximately 50% inhibition of PLA_2 activity *in vitro* was very low ($ED_{50}=2\times10^{-8}$ M).

Based on the observations in Fig. 2 and 3, we exploited melittin's capacity to bind to PLA_2 in developing an enzyme-linked assay for PLA_2 quantitation. In this procedure, binding of biotinylated synthetic melittin to streptavidin-peroxidase is inhibited by the PLA_2 content of synovial fluid. PLA_2 present in the biological fluid binds with biotinylated melittin. When a fixed amount of streptavidin-peroxidase is added, it reacts with only available biotinylated melittin. The decrease in color development is indicative of the presence of PLA_2. ELISA methods for quantitative assays for secretory PLA_2 have been reported (19, 20), and require generation of anti-PLA_2 antibodies and the use of a

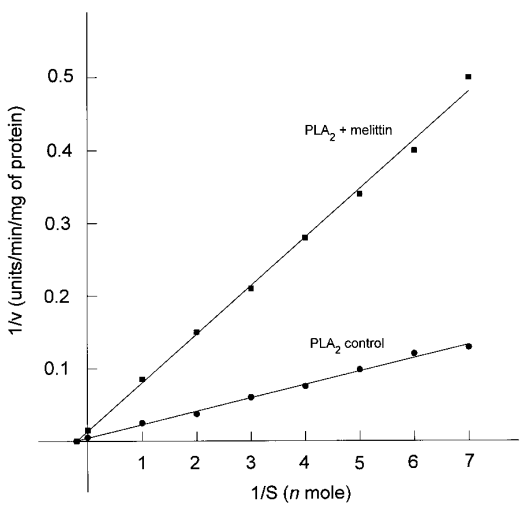

FIG. 2. Lineweaver-Burk plot showing bee venom PLA_2 activity, as determined with [^{14}C]-arachidonic acid-labeled phosphatidylcholine performed in the presence (■) and (1μg/ml) and in the absence (●) of melittin. This graphic presentation is typical of a noncompetitive inhibition reaction. The K_m in both cases remained the same (0.75 nm), while V_{max} decreased from 200 to 50 U/min/mg of protein.

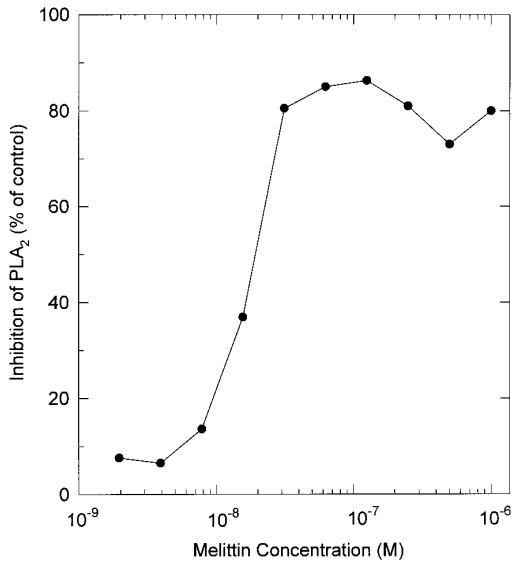

FIG. 3. The molar concentrations of melittin required for maximal inhibition of bee venom PLA_2 activity when [^{14}C]-arachidonic acid-labeled phosphatidylcholine was used as substrate. The concentration of PLA_2 in this assay was 1 nM, and the amount of melittin required to inhibit maximal PLA_2 activity (80%) was 30 nM, a 30:1 ratio of melittin to PLA_2.

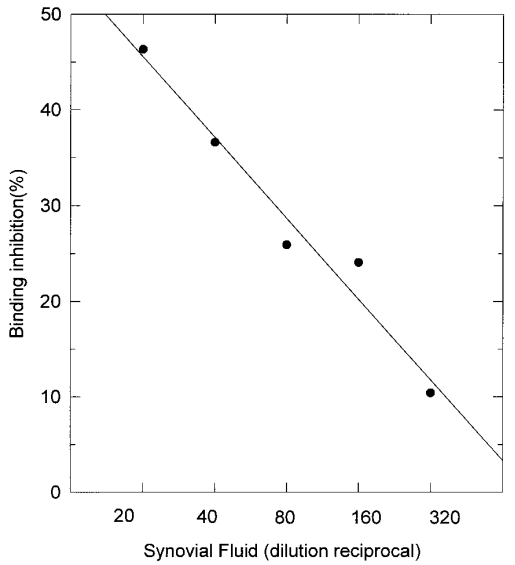

FIG. 4. Enzyme-linked assays based on binding of biotinylated melittin to PLA_2. This enzyme-linked assay for PLA_2 quantitation is based on the binding of biotinylated synthetic melittin to strepavidin-peroxidase and inhibition of this binding by PLA_2 in synovial fluid. Each well of the assay plate was coated with 100 μl of biotinylated synthetic melittin (1 μM) overnight at room temperature. After washing and blocking with 10% BSA, two-fold serial dilutions of synovial fluid were added. After washing, the plates were treated with streptavidin-peroxidase (1:400), and color was developed with ABST substrate. Absorbance of the color reaction was monitored at 405 nm.

second antibody. The assay reported herein appears to be simple and inexpensive. Binding of melittin to PLA_2 has offered yet another procedure to purify and isolate PLA_2 from a variety of biological fluids. Glycine-HCl eluted PLA_2 from a melittin-sepharose column, which had been loaded with human serum (Fig. 5). The resulting purified PLA_2, which showed 1,521-fold purification in terms of specific activity of the enzyme (Table 2A), was inhibited by melittin (Table 2B). The presence of PLA_2 activity in the Centricon 30 retentate suggested that the molecular size of this enzyme was more than 30 kDa. A 45-kDa PLA_2 from human plasma belonging to group VII (21) has been reported (22); however, the PLA_2 isolated from human serum with the melittin-sepharose column needs further characterization.

The observation that melittin inhibited secretory PLA_2 could be related to treatment regimens for rheumatic conditions with bee venom (apiotherapy), an anti-inflammatory method known long before the advent of the medical profession. The first medical report on apiotherapy was published in 1859 (23). Osol and Farrer in 1955 summarized the status of bee venom therapy for arthritis and related conditions in The United States Dispensatory (24). The latest report on the bee venom therapy in arthritis patients appeared in 1966 (25.). Haberman (1972) reviewed work done in his laboratory and others, which compared pharmacologically and biochemically active constituents of various animal venoms, and melittin, a 26-amino acid PLA_2 amphipathic polypeptide (2,840 d), was reported as a major component of bee venom (50% of dry wt) (1). After the report of PLA_2 enhancement by bee venom melittin (2-3), reports on bee venom therapy started to emerge from experimental arthritis studies (26-28). A low molecular weight PLA_2 species was isolated, purified and characterized from synovial fluid and shown to have enhanced PLA_2 activity in rheumatoid arthritis (29). Based on our observations, it could be hypothesized that injection of bee venom into arthritic patients could enable the large amounts of melittin in the venom to bind to synovial fluid PLA_2. Thus formation of melittin-PLA_2 complexes could leave very little free secretory PLA_2 to hydrolyze phospholipids that might other-

TABLE 2A

PLA_2 Activity of Human Serum Chromatographed on Sepharose-Melittin Column as Determined by [^{3}H]-Oleic Acid-Labeled *E. coli* Cells

Source	PLA_2 activity (Units) $\times 10^{-3}$	Purification
Human serum	17.8 ± 1.4	0
Human serum chromatographed on melittin-sepharose	27,066.6 ± 606.0	1,521 fold.

Note. PLA_2 activity was expressed as units, where 1 unit = 1 μmol of substrate hydrolized/min/mg of enzyme.

wise release arachidonic acid, the essential substrate in the formation of proinflammatory eicosanoids. Studies would be needed to test this hypothesis.

In contrast to our observations, two studies using synthetic melittin have shown activation of PLA_2 with PC12 cells (12) and formation of edema in the mouse paw model, which was developed to test the potency of various PLA_2 inhibitors (30). It is possible that melittin *in vivo* may stimulate yet another type of PLA_2 other than secretory PLA_2. Further work is required to identify the PLA_2 type whose activity is enhanced by melittin *in vivo* or *in vitro.*

Based upon the hydrophobic nature of melittin (31), and its capacity to bind to PLA_2, as we have demonstrated, it is possible that melittin could act as a carrier for PLA_2 to translocate it to the membrane. A PLA_2-activating protein (PLAP), whose presence has been shown in various cell types, has been implicated in causing inflammatory disorders (32-37). Murine PLAP has a sequence homology with the "KVLTT" amino acid sequence of melittin (34), and thus could function in a manner similar to melittin. However, further work is required to establish this correlation.

In recent years melittin has been reported to inhibit NA^+/K^+-ATPase, H^+/K^+-ATPase, Ca^{2+} binding proteins, such as protein kinase C, phosphoryl kinase, calsequestrin, calmodulin, and acyl carrier protein (7). Inhibition of Ca^{2+} ATPase by melittin has been shown to be an noncompetitive reaction (38). Further work is required to show if synthetic melittin free of any contamination also has a pleiotrophic nature like that of natural melittin.

Hence we conclude that synthetic melittin inhibits secretory PLA_2 from bee venom, snake venom, bovine pancreas, synovial fluid from rheumatoid arthritis patients and human serum PLA_2 *in vitro.* Inhibition of bee venom PLA_2 by melittin is noncompetitive. Based upon melittin binding to bee venom PLA_2, methods to quantitate PLA_2 in biological fluid and for isolation of PLA_2 by melittin-sepharose-affinity column chromatography have been described. Enhancement/activation of PLA_2 activity *in vivo* by melittin in various cell lines may be due to other types of PLA_2 and not to secretory PLA_2. Melittin or its analogs might have potential value as therapeutic agents against various inflammatory conditions arising from increased formation or activity of secretory PLA_2. Further work is required to identify the phospholipase(s) whose activity is enhanced *in vivo* when cells are exposed to melittin.

TABLE 2B

Effect of Melittin (5 μM) on PLA_2 Activity of Centricon Concentrated/Pass through Samples from Human Serum Eluted from Sepharose-Melittin Column

Samples	PLA_2 activity (nMl/min/ml)	
	Control	MLT
Human serum PLA_2 eluted from MLT-sepharose column centricon 10 concentrated	812 ± 18.2	219 ± 16.3
As 1 but Centricon 30 concentrated (5 times)	696 ± 9.81	192 ± 5.44
As 1 but Centricon 30 pass through	197 ± 13.4	172 ± 8.73

Note. [^{3}H]-Oleic acid-labeled *E. coli* cells were used as substrate.

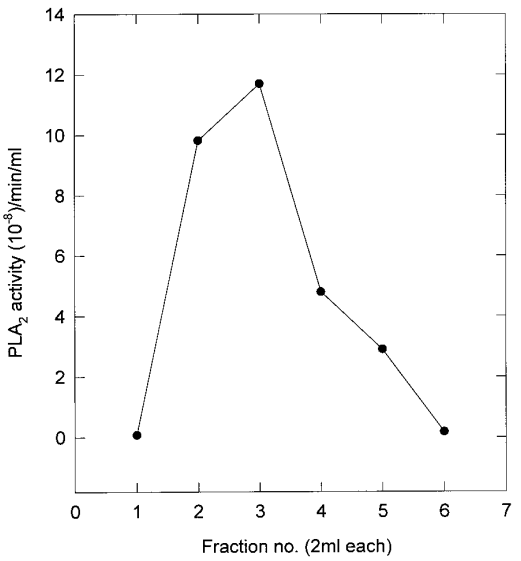

FIG. 5. Chromatography of human serum on melittin-sepharose columns. 10 ml of heat-inactivated human serum was diluted 1:10 with PBS and loaded onto the melittin-sepharose column which was pre-equilibrated with PBS. The column was washed with 500 ml of PBS overnight and eluted with 0.5 M glycine/HCl buffer, pH 2.8. Two-ml fractions were collected, adjusted to pH 7.2 with Tris-HCl buffer, and analyzed for PLA_2 activity with [^{3}H]-oleic acid-labeled *E.coli* cells as substrate.

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