Identification and Isolation of a Mammalian Protein Which Is Antigenically Related to the Phospholipase A2 Stimulatory Peptide Melittin*

Mike A. Clark, Theresa M. Conway, Robert G. L. Shorr, and Stanley T. Crooke

From the Smith Kline and French Laboratories, Department of Molecular Pharmacology, Seldersdale, Pennsylvania 19479

Antibodies prepared against the phospholipase A$_2$ stimulatory peptide melittin were used to identify and isolate a novel mammalian protein with similar functional and antigenic properties. The mammalian protein of $M$, 28,000 was isolated from cell sonicates by high performance immunoaffinity chromatography and size exclusion chromatography. This stimulatory protein was stable for several months when frozen at $-70^\circ$C. The purified protein selectively stimulated phospholipase A$_2$ when phosphatidylcholine was used as a substrate but had no effect on phospholipase A$_2$ activity when phosphatidylethanolamine was used as a substrate. Furthermore, this protein had no effect on phospholipase C activity or on pancreatic or snake venom phospholipase A$_2$. The stimulatory activity was unaffected by RNase or DNase treatment. However, boiling or trypsin digestion inactivated the phospholipase A$_2$. The stimulatory activity was identified as melittin by using antibodies prepared against this peptide.

Phospholipase A$_2$ (PLA$_2$) is an enzyme thought to play a pivotal role in the proinflammatory production of various eicosanoids (1). During the last decade significant progress has been made in the isolation and characterization of the PLA$_2$ enzyme from a variety of sources (for review, see Ref. 2). Phospholipase A$_2$ may be regulated in a variety of ways. A steroid inducible mammalian protein, "lipocortin," which has been shown to inhibit PLA$_2$ activity, has been identified. The purpose of the present study was to determine if mammalian cells contained a phospholipase A$_2$ stimulatory peptide similar to melittin.

**EXPERIMENTAL PROCEDURES**

* Materials—Synthetic melittin was obtained from Peninsula Laboratories (Belmont, CA). Radioactive phospholipid substrates were from New England Nuclear. Thin layer chromatography plates were from Analtec (Newark, DE). Ultrafiltration EP columns were from Beckman Instruments.

**Cell Culture**—The murine smooth muscle cell line B1/3H, was obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 20% fetal calf serum (Hy Clone, Logan, UT).

**Immunizations**—Melittin (1 mg/ml) was denatured and cross-linked by incubation with glutaraldehyde (3% vol/vol) for 20 min at room temperature. This material was mixed with adjuvant and, 100 $\mu$m was injected into New Zealand White female rabbits (2-4 kg) at 2-week intervals. Rabbits were bled 3 days after second or third boost. Blood was allowed to coagulate overnight at 4°C, and the serum was removed the following morning. Immunoglobulin fractions were prepared by ammonium sulfate precipitation followed by desalting on columns of Sephadex G-25 equilibrated with phosphate buffered saline (PBS), pH 7.4.

**Immunofluorescence**—The fluorescein-conjugated anti-melittin antibody (200 mg/ml) was collected and used in control experiments. Loosely bound protein was removed by washing with PBS (two 2-ml washes) and PBS was used.

**Indirect Immunofluorescence**—The coverslips were placed, cell side down, on 20 ml of PBS, pH 7.4. Purified antibody solution (300 pg/ml) through the column overnight at 0°C. Bound immunoglobulin was washed with PBS, pH 7.4. Purified antibodies were aliquoted and stored frozen at $-20^\circ$C. Indirect Immunofluorescence: Cells were grown on glass coverslips for 3 h, rinsed twice with PBS, and then fixed using 3% formaldehyde in PBS for 20 min at room temperature. Cells were permeabilized by using 0.05% Tween 20 for 5 min and washed twice in PBS before being placed, cell side down, on 20 ml of a 1:100 dilution of antibody. Faint binding antibodies and absorbed were (i.e. immune serum which had all melittin reactive antibodies removed from it by affinity chromatography) were used undiluted. The coverslips were incubated for 30 min at 37°C after which they were rinsed 3 times for 5 min in PBS before being placed cell side down on 20 ml of fluorescein-conjugated goat anti-rabbit IgG (Cappell, West Chester, PA) diluted 1:40 in PBS. Cells were incubated for 30 min at 37°C, rinsed 3 times for 5 min in PBS, and the coverslips were then mounted in glycerol/PBS (9:1, v/v). Fluorescence was viewed using a Zeiss UV-equipped microscope.

**Immunofluorescence**—Purification of PLAP—Affinity-purified melittin antibodies in PBS were immobilized on a Beckman HPLC affinity column by recircling (0.1 ml/min) 1 ml of affinity-purified antibody solution (300 mg/ml) through the column overnight (6). PBS containing 0.05% Tween 20 was used to wash the columns extensively. Columns were then equilibrated with PBS containing the protease inhibitors phenylmethylsulfonyl fluoride (10 $\mu$m), bacitracin, (100 mg/ml), benzamidine (1 mm) and soybean trypsin inhibitor (5 mg/ml), and 0.05 Tween 20. Cell sonicate was then passed through the column at a 0.1 ml/min. The cell sonicate was prepared as follows. Logarithmically growing cells were removed from five 150-cm$^2$ Corning tissue culture flasks (Corning, NY) and concentrated by centrifugation (500 x g for 5 min). Cells were resuspended in 1 ml of...
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Pucks Saline F (GIBCO) containing 10 mM Hepes, protease inhibitors (10 μg/ml of soybean trypsin inhibitor; 1 mM benzamidine; 100 μg/ml of bacitracin, and 10 μM phenylmethylsulfonyl fluoride) and detergents (0.05% Tween 20; 0.04% sodium dodecyl sulfate, and 1 mM deoxycholate) and quickly sonicated using a Branson sonicator. Sonicates were centrifuged in a microfuge for 20 min at room temperature, and the resulting supernatant passed through a 0.2-μm Millipore filter (Millipore Corp., Bedford, MA).

The filtered sonicate was passed through the anti-melittin antibody column at 0.1 ml/min. The column was washed for 30 min at 2 ml/min followed by elution using 50 mM sodium acetate, pH 3.1. Fractions (0.5 ml) were collected and stored frozen at −70°C until use. Columns were re-equilibrated with PBS containing 0.05% Tween 20 and stored at 4°C. Columns were used for at least five purification experiments without loss of efficiency. Aliquots (20 μl) of each fraction were assayed in triplicate for endogenous phospholipase A2 activity and for the ability to stimulate phospholipase A2 activity in cell-free sonicates.

Stimulatory activity was defined as the difference between the activity observed in reactions containing cell-free sonicates and any endogenous activity observed in fractions from the affinity column. One unit of stimulatory activity was defined as that amount of PLAP required to produce a 2-fold increase in the observed PLAz activity found in 1 mg of protein/ml of cell sonicate. PLAP recovered from affinity columns remained stable for several weeks at −70°C and could by thawed and refrozen at least 3 times without noticeable loss of activity.

Size Exclusion Chromatography—PLAP activity eluted from affinity columns was chromatographed through a (0.8 x 20 cm) TSK3000 sw HPLC column equilibrated with PBS containing 0.05% Tween 20, at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected. The molecular weight standards (detected by absorbance at 280 nm) used to calibrate the column were phosphorylase B (94,000 Mr), bovine serum albumin (67,000 Mr), ovalbumin (43,000 Mr), carbonic anhydrase (30,000 Mr), soybean trypsin inhibitor (22,500 Mr), and lysozyme (14,000 Mr) (Bio-Rad). Fractions from the size exclusion column were assayed for stimulatory activity as described above.

Assays—Phospholipase A2 and phospholipase C activities were quantitated radiometrically as previously described (8,9) except that reactions were buffered with 200 mM Tris, pH 9.0. Protein concentrations were assayed by the method of Bradford (10) using bovine serum albumin as a standard.

Electrophoresis—Polyacrylamide gel electrophoresis was performed according to Laemmli (11). Protein samples were iodinated with 125I-Bolton-Hunter reagent (New England Nuclear) or by the chloramine-T method using Iodo Beads (Pierce Chemical Co.) as described by the manufacturers. Unreacted 125I-Bolton-Hunter, or free iodine, was removed by desalting on Sephadex G-10 (Bio-Rad) columns equilibrated with 10 mM Tris, pH 7.6, 10 mM EDTA, and 0.1% sodium dodecyl sulfate. Gels were dried and protein bands visualized by autoradiography using Kodak XAR-5 x-ray film.

RESULTS

Indirect Immunofluorescence—To visualize and localize antibody binding at the cellular level, smooth muscle cells were stained using affinity-purified melittin antibody and fluorescent second antibody (Fig. 1A). As shown, the cytoplasm of these cells stained diffusely, and no staining was observed in the nuclei. Little staining was observed if cells were not first permeabilized with low concentrations of Tween-20 (data not shown). Absorbed sera, i.e. immune serum from which the melittin-binding component had been absorbed (Fig. 1C) and preimmune serum (Fig. 1B) failed to stain the cells. From these data we conclude that the mammalian cells contained antigenic determinants similar to those of melittin.

PLAP Isolation—Immunofluorescence affinity chromatography was used to detect the presence of a mammalian protein antigenically similar to the bee venom peptide melittin. In B and C, preimmune serum and immune serum and immune sera which had been depleted of melittin-binding activity were used.
phospholipase A₂ activity was typically 8 ± 2.0 pmol of product produced per minute per milligram of protein. A typical chromatogram is shown in Fig. 2. As shown, a peak of stimulatory activity (10 units/20 μl) was associated with fractions 17 and 18. In control experiments affinity columns were prepared using preimmune serum; these columns failed to retain any phospholipase A₂ stimulatory activity (data not shown). Fraction 18 was then chromatographed on a size exclusion HPLC column. Aliquots (20 μl) from each fraction collected from the size exclusion column were assayed in triplicate for endogenous and stimulatory activity. Fig. 3 shows that PLAP activity appears to chromatograph as a protein of 40,000 kDa. Fractions were iodinated using [125I]-Bolton-Hunter reagent or chloramine T, and polyacrylamide gel electrophoresis was performed to estimate the purity of the resulting material. The starting material, fraction 18 of the affinity column and fraction 27 from size exclusion chromatography are shown in Fig. 4. Fraction 27 from the size exclusion column was used in all of the remaining experiments. The major protein found in the fraction containing the stimulatory activity had a 28,000 kDa. Furthermore, this fraction did not contain measurable (>0.1 pmol of PLAP₂ activity in 20 μl) endogenous activity. Because detergents were used in the mobile phase of the size exclusion chromatography and because of the possibility of the proteins interacting with the column matrix, the apparent differences in the molecular weight, as determined by these different techniques, are probably not significant. The other proteins found in fraction 18 from the affinity column did not appear to react with anti-melittin antibody, and their identity is at present unknown.

**Protein-associated PLAP Activity**—To determine if the phospholipase stimulatory activity was a protein, 50-μl aliquots of PLAP were treated with either trypsin (10 μg/ml), DNase (30 μg/ml), or RNase (30 μg/ml) for 30 min at 37 °C. To quench the trypsin reaction, a 5-fold molar excess of soybean trypsin inhibitor was added. (This amount of soybean trypsin inhibitor did not affect the phospholipase activity assay (data not shown).) Additional 50-μl aliquots were boiled for 10 min. After these treatments stimulatory activity was assayed in triplicate. RNase and DNase treatment did not affect the stimulatory activity, but trypsin digestion and boiling destroyed activity (Table I).

**Specificity of PLAP Activity**—To determine if the PLAP stimulatory activity was specific for phospholipase A₂, 10 units of PLAP were added to cell sonicates and both phospholipase C and phospholipase A₂ activities against various substrates were assayed. As shown in Table II, only the phosphatidylcholine-specific PLAP₂ activity increased. Furthermore, phospholipase C activity was not altered by the addition of PLAP to the assay. In subsequent experiments PLAP did not increase a pancreatic phospholipase A₂ or phospholipase C isolated from crotalus or apis venom (data not shown).

To determine if PLAP increased phospholipase A₂ activity by altering the Kₘ or the Vₘₐₓ of the enzyme, kinetic experiments were performed (Fig. 5). We observed an increase in the apparent Vₘₐₓ of the activity that was dependent on the amount of PLAP added to the reaction, the apparent Kₘ remained unchanged. However, because the phospholipase A₂ activity was so low, it was not possible to perform a series of experiments with varying concentrations of PLAP to see if this effect could be reversed.

![Fig. 4. Polyacrylamide gel electrophoresis of PLAP.](image-url)
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Table I

| Treatment          | Smooth muscle cells | pmol substrate hydrolyzed/min/mg protein |
|--------------------|---------------------|---------------------------------------|
| Sonicate only      | 1.0 ± 0.2           |                                       |
| PLAP only          | <0.1                |                                       |
| Sonicate + PLAP    | 24.0 ± 3.1          |                                       |

Table II

| Activity            | Phospholipase C | PLAP |
|---------------------|-----------------|------|
|                     | PC              | PE   |
| Ssonicate only      | 4.9 ± 0.6       | 8.2 ± 0.9 |
| PLAP only           | <1              | <1   |
| Sonicate + PLAP     | 3.6 ± 0.7       | 46.0 ± 0.3 |

FIG. 5. Lineweaver-Burk analysis of phospholipase A₂ activity as a function of PLAP concentration. Increasing concentrations of PLAP in units: 0 units, Kₘ = 49 Vₘₐₓ = 45; 0.5 units, Kₘ = 33 Vₘₐₓ = 64; 1.0 units, Kₘ = 45 Vₘₐₓ = 106; 1.5 units, Kₘ = 41 Vₘₐₓ = 131; 4.0 units, Kₘ = 50 Vₘₐₓ = 222; 6.0 units, Kₘ = 64 Vₘₐₓ = 192 were added to the cell-free sonicate (1 mg/ml), and phospholipase A₂ activity was assayed using increasing concentrations of phosphatidylcholine as a substrate. Assay conditions were described under "Experimental Procedures". The data shown represents results from two experiments with each data point assayed in triplicate. The Kₘ values are expressed as μM; Vₘₐₓ values are pmol/min/mg of protein.

Discussion

Bee, wasp, and hornet venom peptides have a well-characterized ability to stimulate phospholipase A₂ activity. To determine if a similar mammalian protein existed, antibodies were prepared to glutaraldehyde-cross-linked melittin, and these antibodies were used to detect and purify cross-reacting proteins in mammalian cells. The results from the indirect immunofluorescence experiments suggested that affinity-purified melittin antibodies were able to recognize antigenic determinants in mouse smooth muscle cells.

Immunofluorescence chromatography was used to isolate cross-reacting material from cell sonicates. Phospholipase stimulatory activity appeared to be associated with this material. The stimulatory activity was abolished by boiling or by addition of trypsin, but no loss of activity was observed when this material was incubated with DNase or RNase. These experiments suggest that the stimulatory activity is a protein. Although the stimulatory protein isolated here was much larger than bee venom melittin (2800 Mr), it is apparently antigenically related. We have termed the mammalian cellular protein "phospholipase-activating protein" and report here its partial characterization.

We are now attempting to identify the other proteins which contaminate the PLAP obtained from the affinity purification. Only the 28,000 Mr protein appears to be recognized by the anti-melittin antibody as determined by immunoblotting techniques. The lower molecular weight material may correspond with the small amount of endogenous phospholipase A₂ activity found in the affinity-purified material as it is the approximate molecular weight (=15,000) of several phospholipase A₂ enzymes; however, any endogenous phospholipase A₂ activity we recover by affinity purification of PLAP appears to be unstable and is not detected in the fractions eluted from the size exclusion chromatography. If this smaller protein is phospholipase A₂, it would suggest that PLAP binds to the phospholipase A₂. Although this is an appealing possibility, we do not currently have data to support this. The identity of the higher molecular weight proteins is unknown, and they are not recognized by the anti-melittin antibody. We have also attempted to use the anti-melittin antibody to inhibit the stimulatory activity of PLAP without success. There are at least two possibilities which would explain this. The first is that the antibody-PLAP interaction is disrupted by the detergent used in the phospholipase A₂ assay. The second is that the antibody-binding sites on PLAP are not involved in the activation of phospholipase A₂. The experimental data we currently have is unable to distinguish between these two possibilities.

Under the assay conditions, PLAP stimulatory activity appeared to be specific for phospholipase A₂. It is of interest that phospholipase A₂ stimulatory activity is only observed with phosphatidylcholine as substrate. These results suggest that PLAP activity is specific for a particular subset of phospholipase A₂ enzymes. The mechanism by which PLAP increases phospholipase A₂ activity appears to involve an increase in the Vₘₐₓ of the enzyme.

In these experiments, the largest number of cells used to isolate PLAP was 10⁶. The total amount of protein recovered was <0.1 μg. Because we have defined stimulatory activity as the difference between the endogenous phospholipase A₂ activity in the fraction and the stimulated activity seen in the sonicate plus PLAP, starting material, by definition, had no stimulatory activity. Therefore, it is impossible to estimate...
the enrichment of PLAP obtained. However, we were able to recover 80% of the stimulatory activity isolated from the affinity column by size exclusion chromatography, and the final yield of PLAP was $0.5 \times 10^3$ units of activity from $10^8$ cells.

The mechanism by which PLAP increases phospholipase A₂ activity appears to involve an increase in the apparent $V_{\text{max}}$ of the enzyme. These data could be interpreted to mean that PLAP is involved in converting inactive enzymes into an active form. One possible mechanism by which this could occur is by a proteolytic activation of the phospholipase A₂, as has been shown to occur for pancreatic phospholipase A₂ (12). Alternatively, it may be that PLAP competes for the same regulatory site on phospholipase A₂ as does the inhibitory protein lipocortin. Another possibility is that PLAP could interact with the substrate and then enhance phospholipase A₂ activity. Experiments are currently in progress to determine which of these possibilities is correct.

In conclusion, a cellular protein (PLAP) which is antigenically related to melittin has been demonstrated in mammalian cells and purified to near homogeneity. We hypothesize that this protein is important in phospholipase A₂ regulation and may be involved in inflammatory responses. Recently, we have found that both the bovine endothelial cell line CPAE, the human monocytic cell line U937, and the murine T-cell line EL4 all contain PLAP. In fact the latter two cell lines appear to contain more PLAP than the smooth muscle cell line used here. In addition we have found that LTD₄ increases PLAP synthesis in the BC₃H₁ and CPAE cells, tumor necrosis factor increases PLAP synthesis in the CPAE cells, and interleukin 1 increases PLAP synthesis in the EL4 cells.²

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