Vol. 140, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1986 Pages 114-119

ANTIBODIES PREPARED TO BACILLUS CEREUS PHOSPHOLIPASE C CROSSREACT WITH A PHOSPHATIDYLCHOLINE PREFERRING PHOSPHOLIPASE C IN MAMMALIAN CELLS

Mike A. Clark*, Robert G.L. Shorr*, John S. Bomalaski†

*Smith Kline & French Laboratories,
†VA Medical Center,
Medical College of Pennsylvania, and
University of Pennsylvania, Philadelphia PA 19104

Received August 26, 1986

SUMMARY: Antibodies against Bacillus cereus phospholipase C were prepared in rabbits and used to affinity purify a phosphatidylcholine-preferring phospholipase C from a human monocytic cell line. Affinity chromatography resulted in an approximately 3000-fold, one-step enrichment of phospholipase C. The human enzyme had an apparent molecular mass of 40,000 daltons as determined by SDS gel electrophoresis. Western blotting analysis demonstrated that this protein interacted specifically with the rabbit antibody raised against bacterial phospholipase C. The purified enzyme preferred phosphatidylcholine as a substrate, was neutral pH active and was inhibited by EGTA. These studies demonstrate that antibodies raised against bacterial phospholipase C may be useful in purifying phospholipase C from a human source.

Phospholipase C is an important enzyme which cleaves the polar head group from phospholipids, producing diacylglyceride. Phospholipase C has been isolated from a number of bacteria and is well characterized (for review see 1). The major phospholipase C isolated from Bacillus cereus appears to prefer phosphatidylcholine as a substrate (2) as does a recently isolated canine myocardial phospholipase C (3). We have been interested in phosphatidylcholine preferring-phospholipase C for the following reasons. First, we observed that peripheral blood monocytes and polymorphonuclear leukocytes isolated from patients with rheumatoid arthritis expressed increased levels of phospholipase C activity compared to control cells, especially with phosphatidylcholine as substrate (4,5). These studies led us to hypothesize that this specific phospholipase may be involved in the pathogenesis of rheumatoid arthritis (4,5).

0006-291X/86 $1.50

Secondly, aspirin was found to inhibit phospholipase C activity in vivo and in vitro (5,6). In order to understand this enzyme better, purified enzyme was needed. Previous investigators used standard protein purification protocols and have obtained approximately a 3000-fold enrichment for phosphatidylcholine preferring-phospholipase C from canine myocardium (3). However, for this study we used the novel approach of preparing antibodies to bacterial phospholipase C which were then used to purify a phosphatidylcholine-preferring phospholipase C from human cells by affinity chromatography. These experiments demonstrate that antibodies raised against a bacterial phospholipase may be useful in purifying a similar phospholipase from a human source. The experimental rational was to first produce antibodies to the bacterial phospholipase C. Second the resulting antibodies were then affinity purified. The affinity purified antibodies were then immobilized on a second affinity column which inturn was used to purify mammalian phospholipase C.

MATERIALS AND METHODS

Cells and Cell Culture: The human monocytic U937 cell line was obtained from Georgio Trincheri (Wistar Institute and University of Pennsylvania, Philadelphia, PA). Cells were grown in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (Hy Clone, Logan, UT). All cells were in the log phase of growth at the time of use.

Antibody Production and Purification: Phospholipase C isolated from B. cereus was obtained from Boehringer Manheim (Indianapolis, IN) and cross linked using gluteraldehyde (3% vol:vol) for 30 min at room temperature. The antigen (2000 units per injection) was mixed with an equal volume of Freunds adjuvant and injected intradermally into multiple sites of New Zealand White rabbits at 2 week intervals. The rabbits were bled 3-4 days following the second and third injections and the blood was allowed to clot overnight at 4°. The next day the serum was removed and the immunoglobulin fraction was enriched by precipitation (the result of adding an equal volume of a saturated solution of ammonium sulfate) and affinity purified using a high performance liquid chromatography (HPLC) Ultraaffinity EP column (Beckman Instruments, Palo Alto, CA) as described by the manufacturer.

Affinity Chromatography of Phospholipase C: Another Ultraaffinity EP column was then prepared using the affinity purified antibody. Briefly, 300 µg of affinity purified antibody in 2 ml of phosphate buffered saline (PBS), pH 7.2, (GIBCO, Grand Island, NY) was circulated through another affinity column overnight at a flow rate of 0.2 ml/min. The next day the column were washed extensively using PBS containing 0.05% Tween-20 at a flow rate of 2 ml/min. Cells were concentrated by centrifugation (1000 x g for 5 min), and resuspended in 2 ml of PBS containing 0.05% Tween-20 and protease inhibitors and sonicated. The protease inhibitors included: phenylmethylsulphonylflouride (10 µM), bacitracin (100 µg/ml), benzamidine (1 mM) and soybean trypsin inhibitor (5 µg/ml). The cell-free sonicate was then centrifuged in a microfuge (13,000 x g for 20 min) and the supernatant filtered through a 0.2 µM filter (Millipore, Boston, MA). The filtrate was passed through the affinity column made with anti-PLC antibody (0.1 ml/min). The mobile phase consisted of PBS with 0.05% Tween-20. The column was then washed using the same mobile

phase at a flow rate of 2 ml/min for 10 min and the bound material was then eluted using 50 mM sodium acetate pH 3.1 at a flow rate of 0.5 ml/min. Fractions were collected (2 ml) in tubes containing 200 µl of 10 x PBS and 400 µl of glycerol.

Phospholipase C Assay: Assays were performed as we have previously described using radiolabeled substrate (4-6). Under these conditions all reactions were linear with respect to time and protein concentration (data now shown). The substrates, phosphatidylcholine-2-[^{14}C]-arachidonyl and phosphatidylinositol-2-[^{14}C]arachidonyl, were 48-65 mCi/mM, respectively (New England Nuclear, Boston, MA).

Gel Electrophoresis: Polyacrylamide gel electrophoresis (7) was performed on samples iodinated using Iodo Beads (Pierce Chemical Co., Chicago, IL) as described by the manufacturer. Western blots were performed by electrophoretic transfer of the proteins from the gel (7) to nitrocellulose (Schleicher & Schuel, Keene, NH) using a buffer containing 150 mM glycine, 50 mM Tris and 20% methanol. The nitrocellulose was blocked for 1 hr using TBST (50 mM Tris HCl pH 8.0, 100 mM NaCl and 0.05% Tween-20) plus 20% fetal calf serum. Affinity purified antibody (10 µg/ml) was then diluted 1:100 in TBST and incubated with the filter for 30 min at room temperature (10 ml per 15 x 15 cm filter). The filters were then washed 3 times (5 min each) using 50 ml of TBST then incubated for 30 min in TBST (10 ml + [^{125}I] protein A (10^6 cpm/filter) (Amersham, Arlington Heights, IL). The filters were then washed 3 times (5 min ea) using 50 ml of TBST then air dried.

RESULTS

Affinity Purification of Phospholipase C: Cell sonicate was applied to affinity columns containing anti-phospholipase C antibody. The columns were eluted and the resulting fractions assayed for phospholipase C activity using phosphatidylinositol and phosphatidylcholine as substrates. The amount of phospholipase C activity found in 20 µl of each fraction was determined and

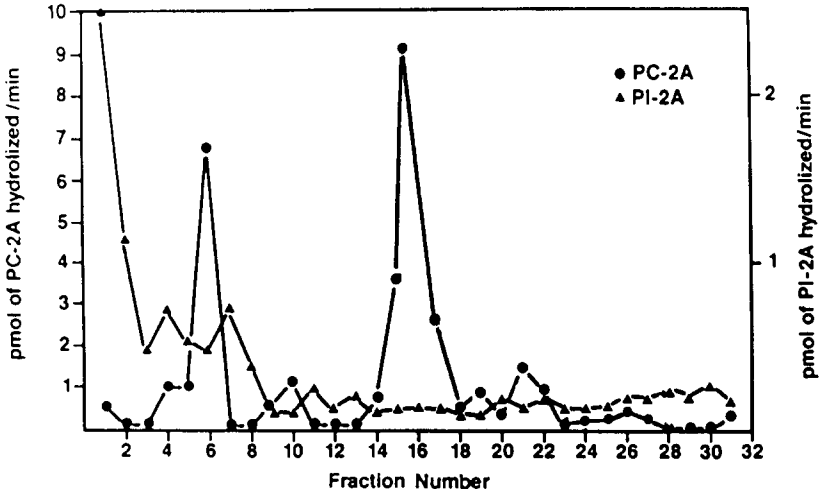

Figure 1. Affinity Chromatography of Phospholipase C. Affinity purified antibodies (~600 mg) were immobilized on a ultra-affinity column. U937 cells (~10^8) were sonicated and applied to the column. The column was then eluted and the resulting fractions were assayed for phospholipase C activity using phosphatidulinositol and phosphatidulcholine as substrates.

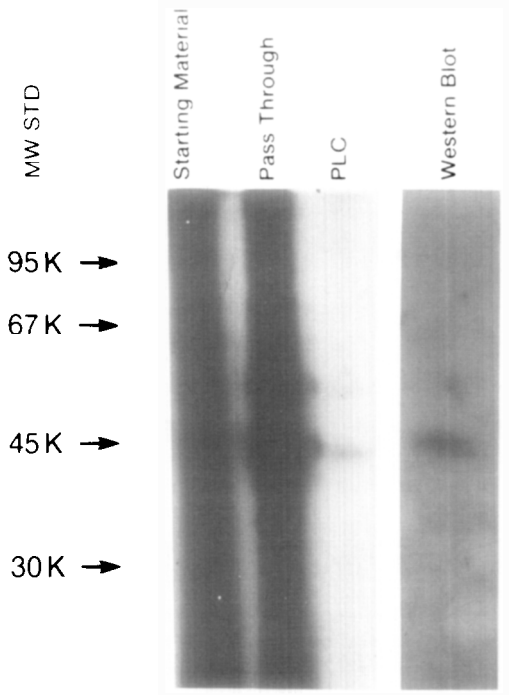

Figure 2. Analysis of Affinity Purified Phospholipase C. The peak fraction (#16 from Fig. 1) from the affinity column was iodinated and compared with the starting material and the flow through from the affinity column. The major protein observed had an apparent molecular weight of 40,000. Western blot analysis indicated that this protein cross-reacted with the antibody prepared to the bacterial phospholipase.

the results are shown in Figure 1. A single peak of activity was observed. From ~10^9 cells (~1 g) a total of 20 µg of protein was obtained. The specific activity of the starting material using phosphatidylcholine as substrate was ~2 pmol of diglyceride produced/min/mg of protein in total cell sonicate. The yield was approximately 3%. The affinity purified material had a specific activity of 7.8 nmol/min/mg of protein, indicating approximately a 3000-fold enrichment. In contrast, the activity observed using phosphatidylinositol as substrate was ~30 pmol of diglyceride produced/min/mg of protein in the whole cell sonicate and the purified material produced less than 1 pmol of diglyceride produced/min/mg.

SDS-PAGE and Western Blotting: To estimate the purity of this enzyme, an aliquot was iodinated and analyzed by gel electrophoresis (Figure 2). Note that the major protein appears to be ~40,000 daltons and nearly twice the size of the bacterial enzyme (23,000 daltons). Western blot analysis of non-

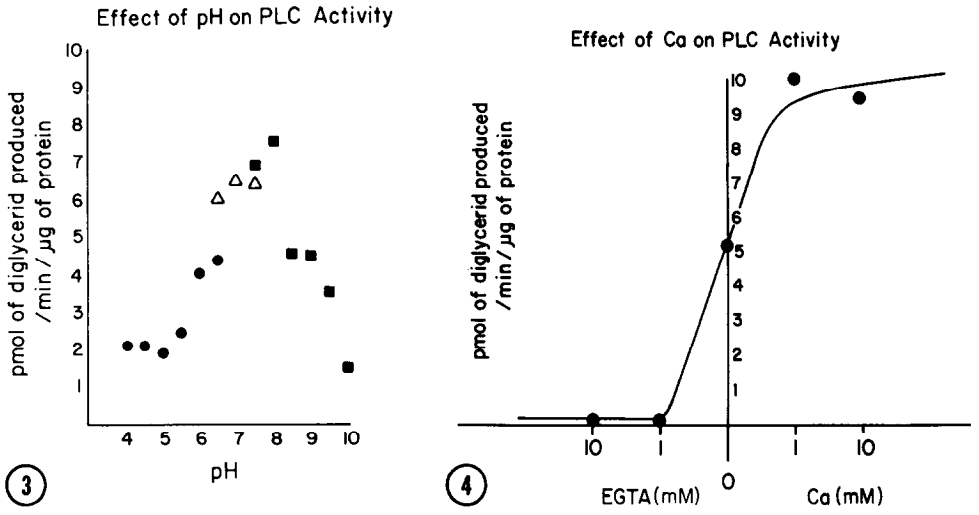

Figure 3. pH Optimization of Mammalian Phospholipase C. The peak fraction (#16 from Fig. 1) from the affinity column was assayed using citrate buffer (X), Hepes buffer (Δ) or Tris buffer (□) at the indicated pH range. All buffers were used at a final concentration of 200 mM.

Figure 4. Calcium Dependency of Mammalian Phospholipase C. The peak fraction (#16 from Fig. 1) from the affinity column was assayed in the presence of the indicated concentration of exogenously added calcium in Tris buffer (pH 7.5). Alternatively the fractions had added to them EGTA 5 min prior to starting the reactions.

iodinated material is also shown (Fig. 2). We observed that antibody produced to the bacterial protein also recognized the 40,000 dalton protein. This protein was unstable and freeze-thawing of the purified material resulted in a decrease of the phospholipase C activity.

It has been previously shown that a similar activity, isolated from dog heart (3), was calcium dependent and had a neutral pH optimum. Therefore, pH optimization experiments were performed. The results are shown in Figure 3. The enzyme isolated from those cells also appeared to be calcium dependent and the addition of EGTA to the reaction mixture also reduced the activity of this enzyme (Figure 4).

DISCUSSION

We have used rabbit antibodies, prepared using bacterial phospholipase C as an antigen, to isolate a human crossreactive protein. The human protein has phospholipase C activity and prefers phosphatidylcholine as substrate indicating that the bacterial and mammalian phospholipase C which prefer phosphatidylcholine are antigenically similar. A similar phospholipase C which has been

purified and identified from dog heart using more conventional biochemical techniques (3). The major difference between our report and the previous one is that the protein obtained from the human monocytic cell line U937 has a molecular weight of ~40,000 daltons as determined by SDS gel electrophoresis, in contrast this protein isolated from dog heart ~29,000 daltons as determined by Sephadex gel filtration. The pH optimum reported here for the human enzyme is nearly identical to the data previously reported for the dog heart. The human phospholipase C requires calcium (is inhibited by EGTA) and appears to be different from the acid pH active, calcium independent (EGTA insensitive), phosphatidylcholine-preferring phospholipase C activity found in rat tissues (8,9).

Other investigators have isolated, from sheep seminal vesicles, at least 2 distinct forms of phospholipase C that hydrolyze phosphatidylinositol and are antigenically distinct (10). In addition, multiple forms of PLC which prefer phosphatidylinositol are present in various rat tissues (11). These data, taken as a whole, indicate that there may be several different PLC enzymes present in mammalian cells, some of which may have specificity for individual phospholipid classes. Perhaps one of the reasons that only 3% of the PLC activity was recovered is that these antibodies only recognize one subclass of the phosphatidylcholine PLC enzymes. These enzymes may play multiple roles in the regulation of eicosanoid production and receptor-mediated signal transduction.

REFERENCES

1. Dennis, E.A. (1983) in The Enzymes (P.d. Boyer, ed.), Vol. 16, Academic Press, New York, pp. 307-353.
2. Otnaess, A.B., Little, C., Sletten, K., Wallin, R., Johnsen, S., Flangsrud, R. and Prydz, H. (1977) Eur. J. Biochem. 79:459-468.
3. Wolf, R.A. and Gross, R.W. (1985) J. Biol. Chem. 260:7295-7303.
4. Bomalaski, J.S., Clark, M.A., Douglas, S.D. and Zurier, R.B. (1985) J. Leuk. Biol. 38:649-654.
5. Bomalaski, J.S., Clark, M.A. and Zurier, R.B. (1986) Arthritis Rheum. 29:312-318.
6. Bomalaski, J.S., Hirata, F. and Clark, M.A. (1986) Biochem. Biophys. Res. Comm. (in press).
7. Laemmli, U.K. (1970) Nature (London) 227:680-685.
8. Hostetler, K.Y. and Hall, L.B. (1980) Biochem. Biophys. Res. Comm. 96:388-393
9. Matsuzawa, Y. and Hostetler, K.Y. (1980) J. Biol. Chem. 255, 646-652.
10. Hofmann, S.L. and Majerus, P.W. (1982) J. Biol. Chem. 257, 6461-6469.
11. Low, M.G. and Weglicki, W.B. (1983) Biochem. J. 215:325-334.