Lipase Activity of Phospholipase C from *Clostridium welchii* and *Bacillus cereus* on Cultured Chick Embryo Muscle Cells

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Introduction

Embryonic chick muscle cells fuse in culture and thus provide an excellent system for the study of the molecular basis of cell-cell fusion (1), although the molecular mechanisms by which it is mediated are not yet known. The process of cell fusion in chick myoblasts involves plasma membrane fusion. This process may involve proteins, lipids, or carbohydrates of plasma membranes. Interest has recently focused on proteins and lipids. However, the literature on this subject (proteins - 2, 3, 8, 9, 10, 11, 12, 13, lipids - 4, 5, 6, 7, 14, 15) is contradictory. Recently, inhibitors of cultured muscle cell fusion, phospholipase C from *Clostridium perfringens* and *Clostridium welchii* (PLCC), have been used by several investigators (10, 11, 12, 16).

Because of the potential for imparting specificity with respect to the lipid composition changes associated with PLCC inhibition of muscle cell fusion, a comparison of the lipase activity of PLCC and phospholipase C from *Bacillus ceveus* (PLCB) on cultured chick embryo muscle cells was undertaken.

Methods

Cell Culture

Eleven-day Leghorn chick embryo breast muscle cells were isolated as described (16). The cells were grown in 1621 medium (a complete growth medium consisting of 82.5% Earl's MEM, 10.3% horse serum, 5.2% 11 day chick embryo extract, 1% fungizone, and 1% penicillin-streptomycin solution). Dissociated (trypsin) single cells were plated on gelatin coated 35mm tissue culture dishes (Corning) at 0.3 x 10^6 cells in one ml of 1621 per dish. The culture medium was changed at 24 hours and every other day thereafter. The cultures were incubated in a water saturated atmosphere in 5% Co_2 at 37.5°C. At appropriate times the cultures were fixed in ethanol-formalin-acetic acid (20:2:1) and stained with Myers Hematoxylin. The cultures were mounted directly after staining by adding 1 drop of polyethylene glycol (6,000 MW) to the culture dish and covering the cells with a coverslip. The fusion percentage—defined as the % of cell nuclei within clearly observable multinucleated cells (greater than 2 nuclei)—was determined as follows: 300 to 500 cell nuclei were counted per dish by randomly moving the stage and counting all the nuclei within each field.

Biochemicals and Assays

Phospholipase C from *Clostridium welchii* (PLCC) (type IX, partially purified, specific activity 140 units per mg protein) and phospholipase C from *Bacillus cereus* (PLCB) (type III, specific activity

120 units per mg protein), purchased from Sigma Chemical Co., were used without further purification. Both PLCC and PLCB were suspended in 10 mM phosphate buffer (pH 7.0) and 50% glycerol, sterilized by Millipore filtration (0.22μ), and stored at -10°C. The stock enzymes in this form retained their specific activity for the duration of the experiment. The phospholipase C activity of PLCC and PLCB was assayed by determining the ability of the enzymes to hydrolyse phosphorylcholine-(methyl-^{14}C) from phosphatidylcholine-(choline-methyl-^{14}C) after Nameroff *et al.* (11) with the following modifications: 1) The assay buffer was Earls' MEM at pH 7.2. 2) The carrier lecithin added to the reaction was L-a-phosphatidylcholine dipalmitoyl purchased from Sigma. Phospholipase C activity is defined as follows: one unit of activity releases 1.0 μmole phosphorylcholine from L-a-phosphatidylcholine per minute, at pH 7.2, at 37°C. Choline chloride-(methyl-^{14}C) with a specific activity of 53.5 mCi/mmole and phosphatidylcholine-(choline-methyl-^{14}C) with a specific activity of 53.0 mCi/mmole were purchased from New England Nuclear, Boston, Mass. Whatman LHP-K TLC plates were purchased from Whatman Inc., New Jersey.

Lipase Activity of PLCC and PLCB on Cells

In the beginning of the second day ^{14}C-choline chloride was added to the cultures at a concentration of 1.0 μCi per ml of medium. At 48 to 50 hours (24 hours in the presence of label) the cultures were chased with fresh medium containing 0.2 mM cold choline chloride for 30 minutes. The cultures were then washed 2X with MEM, and incubated with PLCC or PLCB, in MEM, at 37°C for 30 minutes. Samples of the supernatant (MEM) were then collected, centrifuged at 1500 g for 10 minutes, and 100 μl aliquots were counted directly in Dioxin based scintillation fluid or streaked on Whatman LHP-K TLC plates. The TLC plates were developed in glacial acetic acid-n-propanol-water-phenol (1:2:1:1) (19). The standards for choline, phosphorylcholine, and lecithin were run on separate slots on the same plate. The standards were visualized by Dragendorff reagent (20). ^{14}-C-choline and ^{14}C-phosphatidylcholine were also run as controls. After removal of the supernatant from the cells, the cells were scraped from the culture dish and centrifuged into a pellet at 1500 g for 5 minutes. The cell pellet was then immediately extracted with chloroform/methanol (2:1; v/v). 100 μl of the crude lipid extract was chromatographed as described above. After development the TLC plates were air dried, and 0.5 cm strips of the gel were scraped from the plate, added to toluene based scintillation fluid, and counted in a Beckman Scintillation Counter.

Results

Fusion Inhibition Activity of PLCC and PLCB

After the phospholipase C activity of PLCC and PLCB was determined, appropriate dilutions of the enzymes were added to 24 hour old chick embryo muscle cultures to achieve enzyme activities ranging from 1.25 to 750 mU/ml of 1621 medium. At 72 hours the cultures were fixed and stained. Control cultures achieved a fusion percentage

of between 58 and 76 (Fig. 1a). PLCC inhibited fusion from 12.5 to 450 mU/ml (range of 38X) with a fusion percentage of 5 to 15 (Fig. 1b). At 500 mU/ml the number of cells per culture was greatly reduced (Fig. 1d). The fusion percentage at 500 mU/ml was not determined, because it was not known, if there was selective detachment of myoblasts or multinucleated muscle cells, or inhibition of proliferation. At 750 mU/ml no viable cells were detected on the culture dish. In contrast, PLCB did not inhibit fusion over the whole range of activities tested with a fusion percentage of 53 to 78 (Fig. 1c). Although PLCB did show evidence of cell detachment at above 450 mU/ml of activity, cell fusion was still evident (Fig. 1e). Cultures exposed to 100 mU/ml

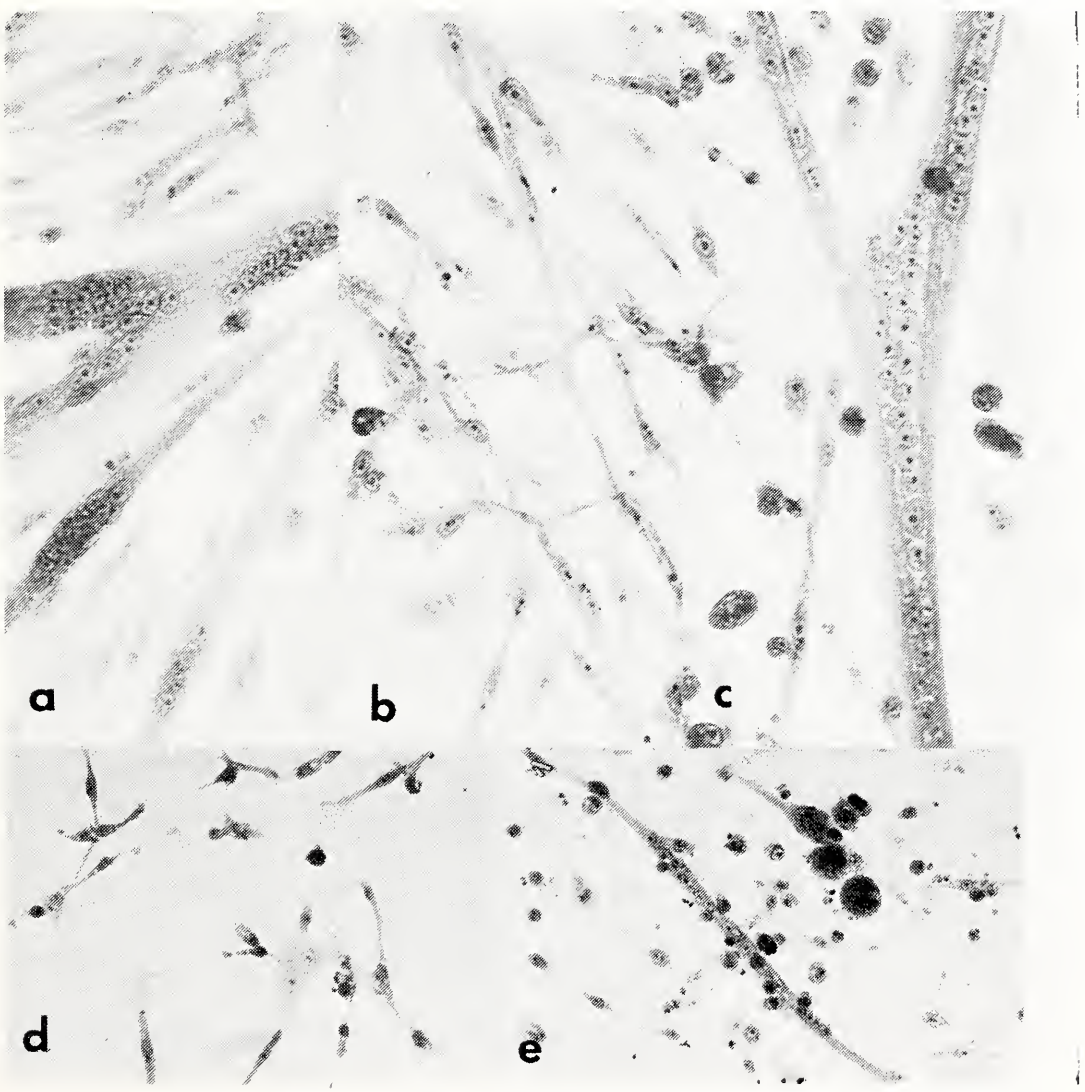

FIGURE 1. *Effects of PLCC or PLCB on chick embryo muscle cell fusion. Cultures were initiated as indicated. At 24 hours varying amounts of enzymes of known phospholipase activity were added to the cultures. The cultures were fixed and stained at 72 hours. (a) Control culture incubated in 1621 only; (b) Culture with 250 mU/ml of PLCC; (c) Culture with 250 mU/ml of PLCB; (d) Culture with 500 mU/ml of PLCC; (e) Culture with 500 mU/ml of PLCB. Figures a, b, and c, are 2X the magnification of figures d and e.*

or more of PLCB showed distinctive rounding up of single cells (Fig. 1c). We concluded that PLCB in contrast with PLCC did not inhibit muscle cell fusion.

Lipolytic Activity of PLCC and PLCB on Cultured Muscle Cells

To determine the effects of PLCC and PLCB on cell lipids the following experiments were performed. After 24 hours in culture, the cultures were labeled for 24 hours with 1 μCi of ^{14}C-choline. After 24 hours of labeling, 85 to 95% of the ^{14}C counts were associated with the lecithin spot as determined by standard TLC chromatography. At 48 hours the cultures were washed and exposed to PLCC and PLCB over a wide range of activities, 1.25 to 500 mU/ml, for 30 minutes, in MEM, pH 7.2, at 37.5°C. Aliquots of the supernatant were counted directly (Fig. 2) and chromatographed (Fig. 3). The cell layer was scraped from the dish, lipids extracted, and aliquots of the lipid extract chromatographed (Fig. 4).

Figure 2 shows that PLCC releases substantial ^{14}C counts into the MEM over a wide range of enzyme activities. The curve generated shows saturation kinetics that is flat over a wide range of PLCC activities. To determine the effect of incubation time on the counts released, several cultures with 25 and 50 mU/ml of PLCC were allowed to incubate for another 30 minutes (1 hour total). Doubling the incubation time only increased the counts released from 10 to 25%. Phase microscopic observations of the cultures after the experiment showed that at all enzyme concentrations, the cells remained attached to the dishes. By 72 hours enzyme treated cultures that were allowed to recover had the same fusion percentage as controls.

To determine the nature of the ^{14}C counts released into the supernatant, 100 μl of the MEM was chromatographed to separate phosphorylcholine, choline, and phosphatidylcholine. Figure 3 shows that nearly all of the counts released by 250 mU/ml of PLCC co-migrates with phosphorylcholine. In agreement with the inability for PLCB to inhibit muscle cell fusion, PLCB does not release ^{14}C counts significantly different from control counts. This experiment was performed with several activities of enzymes with similar results.

To determine the origin of the phosphorylcholine (^{14}C) counts, the cell layer, after enzyme treatment, was lipid extracted and the extract chromatographed to separate phosphorylcholine, choline, and phosphatidylcholine. Figure 4 shows that with the appearance of phosphorylcholine in the supernatant there is a substantial decrease in the phosphatidylcholine associated with the cell layer. This result indicates that the major portion of counts released into MEM by PLCC was the result of the hydrolysis of labeled phosphatidylcholine. PLCB and control curves were not significantly different. PLCB and controls appear to have twice the phosphorylcholine counts associated with the cell layer as the PLCC treated cultures. Since it is not known what proportion of the cell choline and phosphorylcholine were extracted by the lipid extraction procedure, it is not known if this difference is real. We

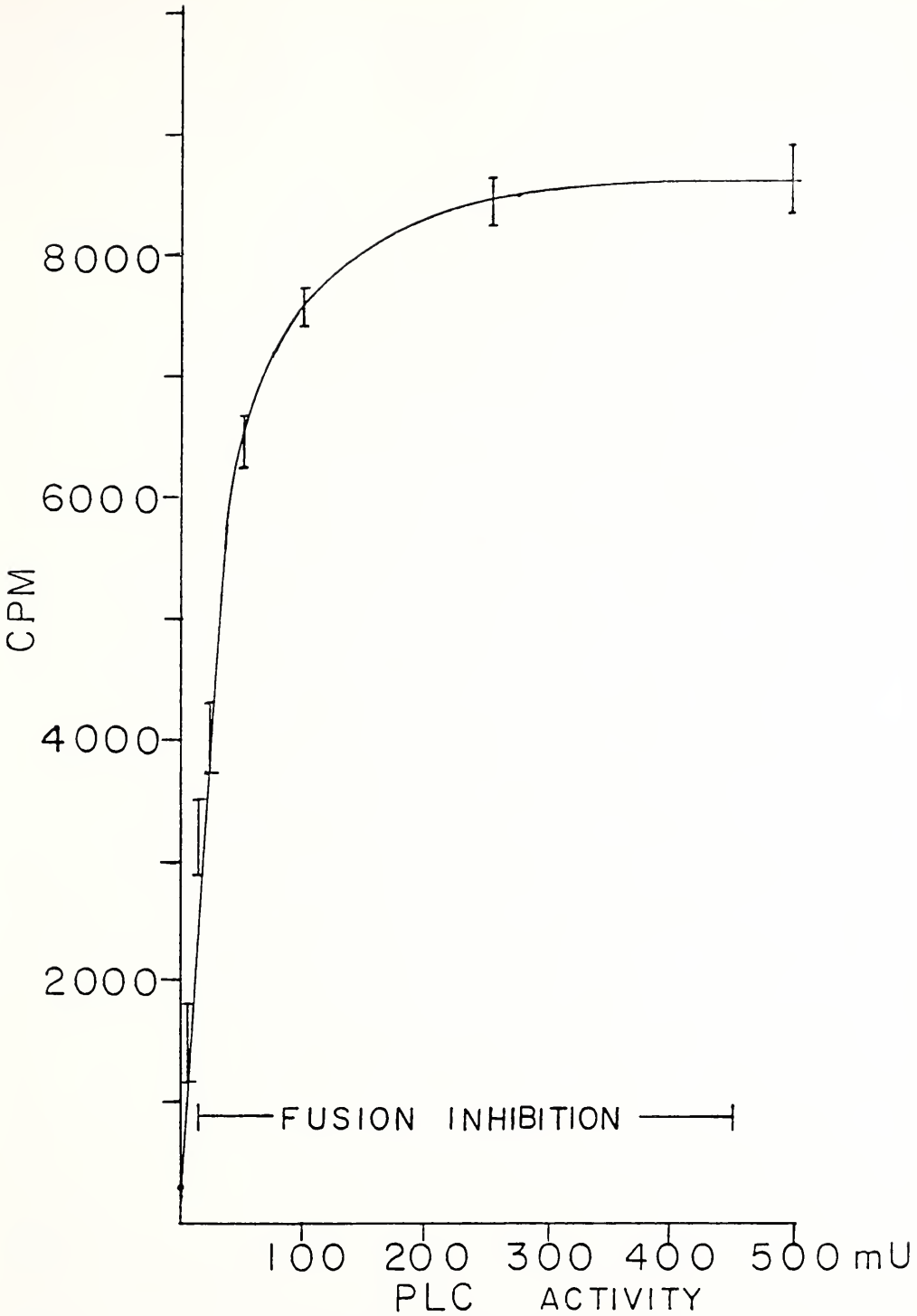

FIGURE 2. *Release of ^{14}C counts from 48 hour old cultured chick embryo muscle cells. Cells were labeled with ^{14}C-choline (1μCi/ml) for 24 hours. Labeled cultures were treated with the dilutions of PLCC equivalent to the phospholipase C activity indicated (1.25 mU/ml to 500 mU/ml) in MEM. Radioactivity was determined by counting 100 μl of the supernatant. The curve generated represents 2 experiments of 2 samples each. The range of fusion inhibition was determined in a separate experiment.*

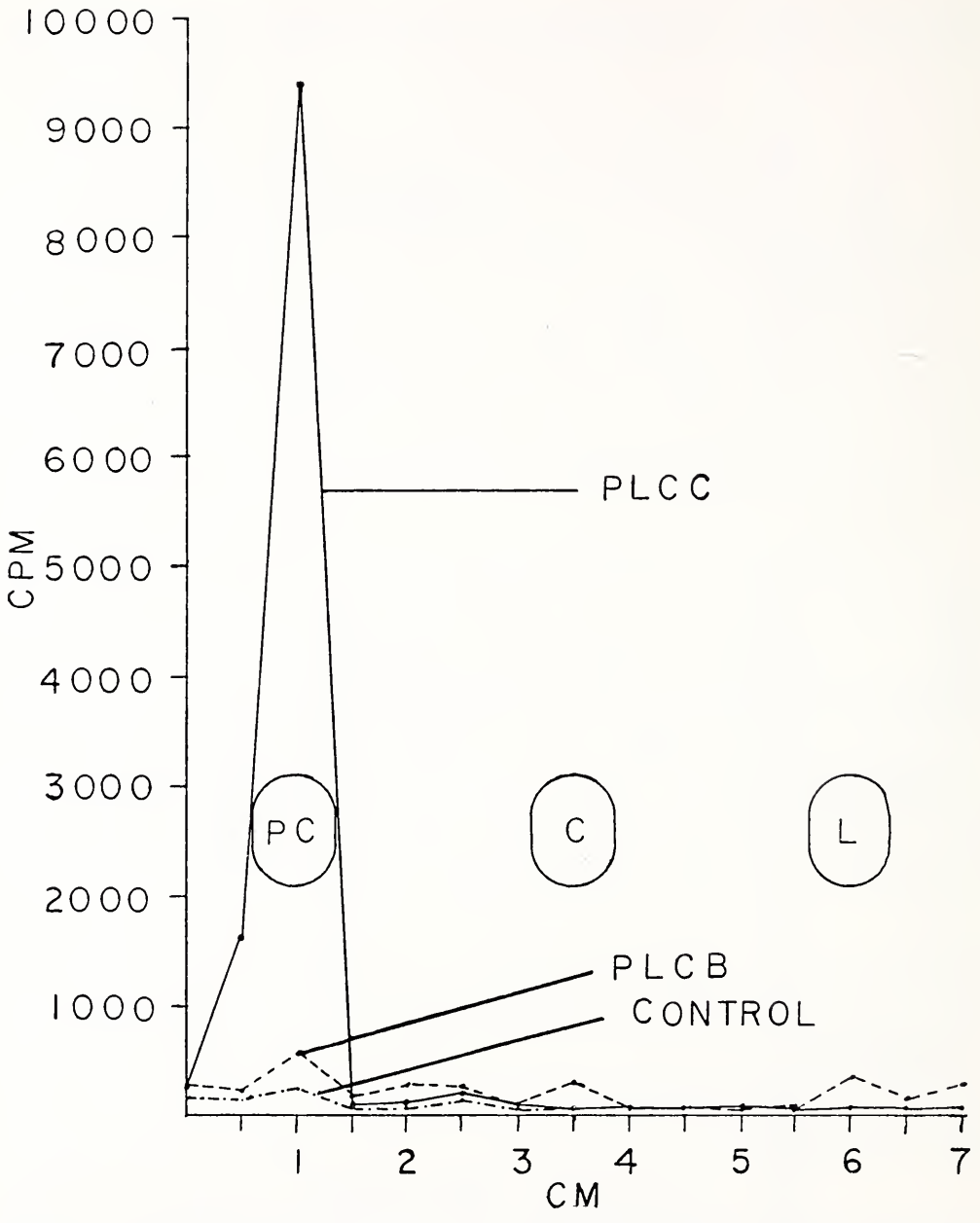

FIGURE 3. *Analysis of the supernatant for the lypolytic effects of PLCC or PLCB. Cells were labeled with ^{14}C-choline (1 μCi/ml) for 24 hours. Labeled cultures were treated with the concentrations of PLCC or PLCB equivalent to 250 mU/ml of phospholipase C activity in MEM. Control cultures were treated with MEM only. Standards (phosphorylcholine-PC, choline-C, and phosphatidylcholine-L) were chromatographed on the same Whatman LHP-K TLC plate but on a separate channel. Radioactivity was determined by counting scraped 0.5 cm strips of silica gel.* ———— *Chromatogram of PLCC;* — — — — — — *Chromatogram of PLCB;* — . — . — . — *Chromatogram of Control.*

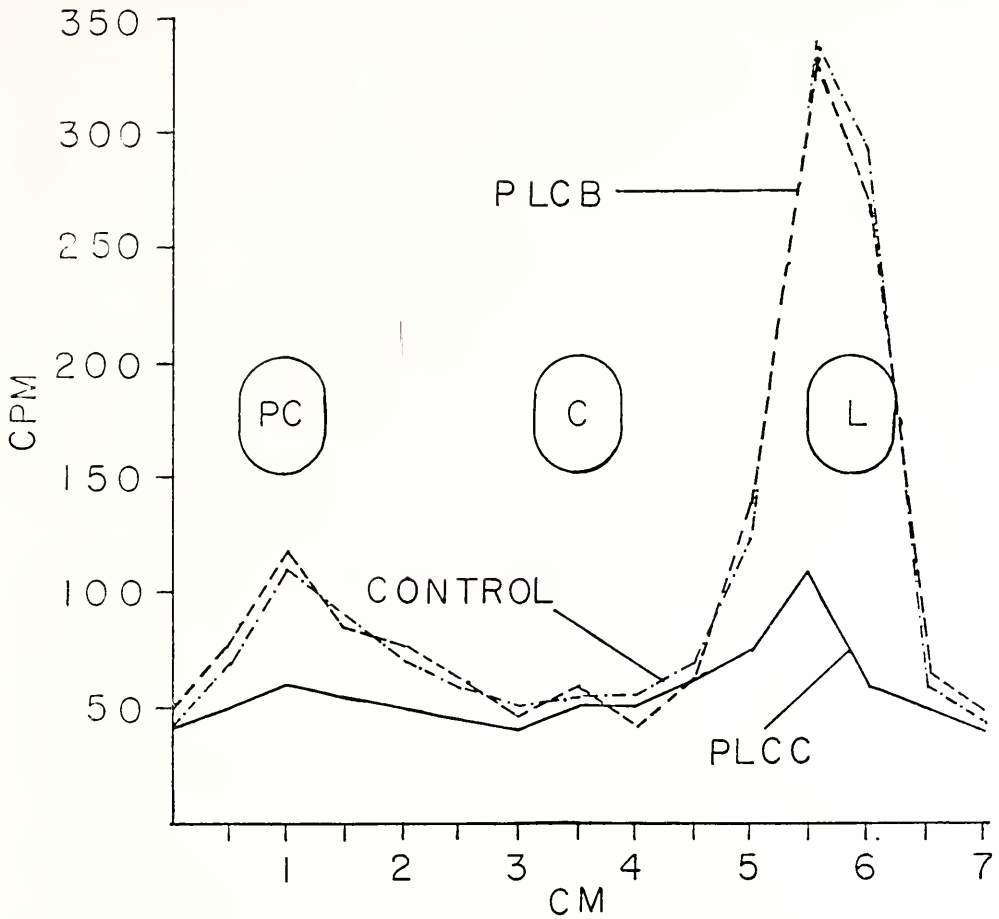

FIGURE 4. *Analysis of muscle cells for the lypolytic effects of PLCC or PLCB. The experimental methods were the same as stated in fig. 3 except the following: After decanting the supernatant, the cell layer was washed in MEM and scraped from the dish. The lipids were immediately extracted with chloroform-methanol and 100 μl of the lipid extracts were chromatographed.* _____ *Chromatogram of PLCC;* — — — — — — *Chromatogram of PLCB;* —.—.—.— *Chromatogram of Control.*

concluded that PLCB in contrast with PLCC did not hydrolyze choline containing phospholipids in the muscle cells.

Discussion

In the present study we have compared the ability of PLCC and PLCB, of equal phospholipase activity, to inhibit chick embryo muscle cell fusion. We have also compared the ability of PLCC and PLCB to hydrolyze chick embryo cell surface choline containing phospholipids. PLCC inhibited fusion over a wide range of enzyme activities, and PLCC treated cultures were able to recover in a short period of time. Published evidence indicates that PLCC does not enter cells (8). The above evidence suggests that the ^{14}C-phosphorylcholine counts liberated are the result of the hydrolysis of cell surface phosphatidylcholine and/or sphingomyelin. It is not known if the reduction in numbers of cells at PLCC and PLCB activities above 450 mU/ml is due to reduced

cell proliferation, cells selectively coming off the substrate, or lysis of the cells. In this regard it is interesting that hemolysis activity in crude preparations of PLCC and PLCB has been reported by other investigators (e.g., 21). The saturation curve in Figure 2 may indicate the following: Phospholipase C activity is not the direct cause of the reduction in cell numbers at the higher enzyme activities. There are only a specific number of sites on the cell surface susceptible to phospholipase C hydrolysis. All of the cell surface choline containing phospholipids have been hydrolyzed.

PLCC hydrolyzes the glycerophosphate bond in several classes of lipids, including sphingomyelin (17). Since we only assayed for the release of phosphorylcholine by PLCC, we are only able to conclude that PLCC hydrolyzes cell surface choline containing phospholipids. The hydrolysis of other phospholipid classes is presently being investigated. Although PLCB does not hydrolyze choline containing phospholipids, it is not known if other phospholipid classes are hydrolyzed.

It has been reported that PLCC (21), but not PLCB (18, 22), hydrolyzes up to 70% of the phospholipids in intact human erythrocytes. On the other hand, pure PLCB together with sphingomyelinase will hydrolyze intact erythrocyte phospholipids (18). This evidence is consistent with the observation that PLCC may have a sphingomyelinase component (17). Similarly, the observation, in our system, that PLCC, but not PLCB, hydrolyzes cell surface choline containing phospholipids, suggests the exciting hypothesis that sphingomyelinase activity may be important in the inhibition of chick embryo muscle cell fusion. This hypothesis is presently being investigated. From this study, we have reached the following conclusions: (a) PLCB, in contrast with PLCC, does not inhibit cultured chick embryo muscle cell fusion; (b) PLCB, in contrast to PLCC, does not hydrolyze cell surface choline containing phospholipids; (c) without further analysis of the effects of PLCB on cell surface lipids, PLCB cannot be used on a comparative basis with PLCC to determine those lipid composition changes that are specific for fusion inhibition by PLCC. (Supported by PHS SO7 RR5371 from the United States Public Health Service.)

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