

Stimulation of phospholipid metabolism in embryonic muscle cells treated with phospholipase C

(phospholipid synthesis/myogenesis)

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ABSTRACT Phospholipid metabolism is dramatically stimulated in cultured myogenic cells in which cell fusion was inhibited with phospholipase C (phosphatidylcholine cholinephosphohydrolase; EC 3.1.4.3). Phospholipase C was active under the culture conditions as shown by the degradation of exogenous phosphatidylcholine. Rates of incorporation of $^{32}\text{P}_i$ and [*methyl*- ^3H]choline into lipids were about 5-fold greater in phospholipase-treated cells than in either untreated fusing cells or untreated cells prevented from fusing by calcium deprivation. The greatest stimulation in the phospholipase C-treated cultures occurred with synthesis of phosphatidylcholine and sphingomyelin; synthesis of phosphatidylinositol and cardiolipin was not stimulated. Degradation of cellular [^{32}P]phosphatidylcholine and appearance in the culture medium of the degradation product [^{32}P]phosphocholine were both increased. Levels of total cellular phospholipids and of individual phospholipid classes were similar in control and phospholipase-treated cells. The results suggest that the membrane phospholipid composition in myogenic cells is controlled by a regulatory mechanism which increases the synthesis of phospholipids that are degraded in the presence of the phospholipase.

Embryonic striated muscle provides an excellent cell culture system for studying the roles of the cell surface membrane in intracellular interactions and embryonic development (1). The plasma membrane of the muscle cell is integrally involved in the processes of cellular recognition, membrane fusion, and differentiation. Although cell surface proteins undoubtedly are indispensable to these processes, plasma membrane lipids are also likely to be involved. The lipid compositions of purified plasma membranes of cultured myoblasts and myotubes (2) have been determined (3). No significant alterations in cell surface lipids were seen at different stages of development. These results, however, do not necessarily rule out the involvement of plasma membrane lipids in muscle development. Myoblast recognition and fusion are prevented by nutritional manipulation of the cellular phospholipid or cholesterol content (4), suggesting that a certain lipid composition must be maintained if the myoblasts are to develop normally.

Another method that has been used to alter cell surface lipids is treatment of myoblasts with phospholipases (5, 6). Nameroff and coworkers (5) reported that addition of phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) from *Clostridium perfringens* to myogenic cultures prevents membrane fusion (5) but not cellular recognition (7). However, the phospholipase C was not shown to be active under the culture conditions or to be degrading phospholipids of the myoblast cell surface (5). The results presented in the present paper demonstrate accelerated hydrolysis of phospholipids in phospholipase-treated myoblasts. Moreover, the phospholipase treatment has striking effects on phospholipid metabolism in myogenic cells.

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MATERIALS AND METHODS

Cultured Cells. Pectoral muscle from 11-day chicken embryos was dissected, loose connective tissue was removed, and the muscle was minced into 1- to 2-mm fragments. Cells were dissociated from the tissue fragments by trituration with a pasteur pipette (8) in calcium- and magnesium-free Earle's salt solution. The cell suspension was filtered through cheesecloth, preplated for 15 min (9), and then diluted with culture medium to 5×10^5 cells/ml. The cells were plated in tissue culture dishes precoated with rat tail collagen (10) at 8 ml of cell suspension per 100 mm dish. Half the culture medium was exchanged for fresh medium on day 1 and the entire medium was exchanged on day 2. Culture medium contained 10 ml of fetal calf serum, 3 ml of chicken embryo extract (2), and 1.2 ml of antibiotic-antimycotic solution per 100 ml of Eagle's minimum essential medium. In experiments in which cells were inhibited from fusing by growth in low calcium medium (11), the composition of low calcium medium was the same as regular medium except that calcium-free Eagle's medium was used and ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was added to chelate calcium contributed by serum and embryo extract. The final calcium concentration in this medium was about 150 μM . Media components were obtained from GIBCO.

At the time of harvest, the culture medium was removed, the cells were washed twice with Earle's salts, and 1.5-2 ml of either H_2O or 10 mM Tris-HCl, pH 7.5, was added. The cells were removed from the dish with a rubber scraper and broken with a Dounce homogenizer. All procedures for harvesting were performed at 4°C. For some experiments, the cell homogenate was diluted to 9 ml with 10 mM Tris-HCl, pH 7.5, and centrifuged at 100,000 $\times g$ for 90 min to obtain a crude particulate fraction.

Lipid Analysis. Lipids were extracted from the cell homogenate or the resuspended crude particulate fraction by the procedure of Bligh and Dyer (12). Phospholipids were separated by one-dimensional (13) or two-dimensional (14) thin-layer chromatography and eluted from the plates as described (3, 15). Egg phosphatidylcholine was purified according to Pangborn (16). Organic phosphate was determined by the procedure of Rouser *et al.* (14).

Phospholipase C. Phospholipase C from *C. perfringens* was purchased from Sigma (type I, 7.4 units/mg of solid) and assayed as described (17). For addition to muscle cultures, phospholipase C was dissolved in calcium- and magnesium-free Earle's salts and sterilized by filtration. Phospholipase C was added to the cultures 24 hr after plating (day 1) and after an additional 24 hr (day 2) when the old medium was removed and fresh medium was added. All experiments were performed with a phospholipase concentration of 22×10^{-3} units/ml.

Phosphoryl Base Analysis. Phosphocholine was analyzed by a modification of the procedure described by Dittmer and

Wells (18). A 4-ml column of AG 1-X8 formate (Bio-Rad) in H₂O was equilibrated with 0.01 M sodium borate, pH 9.7. The sample was eluted first with 10 ml of borate buffer and then with a gradient of borate buffer (12 ml) and 0.65 M sodium formate in borate buffer (12 ml). Fractions of 1.0 ml were collected. This procedure was sufficient to separate choline, phosphocholine, phosphoethanolamine, and P_i. For resolution of these components from phosphoserine and phosphoinositol, the gradient was made with 25 ml of each buffer.

For analysis of phosphocholine in the culture medium, 2 ml of medium was treated with 1 ml of 20% trichloroacetic acid to precipitate serum proteins. Trichloroacetic acid was extracted from the supernatant fraction with diethyl ether. The sample applied to the ion-exchange column consisted of 1.0 ml of ether-washed acid soluble fraction, 0.5 ml of H₂O, and 50 μ l each of 10 mM phosphocholine and 10 mM NaH₂PO₄. Phosphocholine and P_i were located by organic phosphate determination of 50 μ l of each column fraction. For radioactive compounds, a sample of each fraction was added to 3a70 scintillation cocktail (Research Products International, Elk Grove Village, IL) and assayed for radioactivity in a Searle Mark III liquid scintillation counter.

Phosphocholine was also separated from choline and CDP-choline by electrophoresis in pyridine/acetic acid/water, 80:3.2:917, for 30 min at 2000 V. Regions of paper containing labeled compounds were located by scanning with a Packard radiochromatogram scanner, cut out, and assayed for radioactivity in a toluene scintillation fluid.

Miscellaneous Determinations. Protein was determined by the procedure of Lowry *et al.* (19) with bovine serum albumin as the standard. DNA was determined with diphenylamine (20) with calf thymus DNA as the standard. Lactate dehydrogenase was assayed as described (21).

Radiochemicals. ³²P_i was purchased from Schwarz/Mann or Amersham, [methyl-³H]choline chloride was from New England Nuclear, and phosphoryl[methyl-¹⁴C]choline was from Amersham.

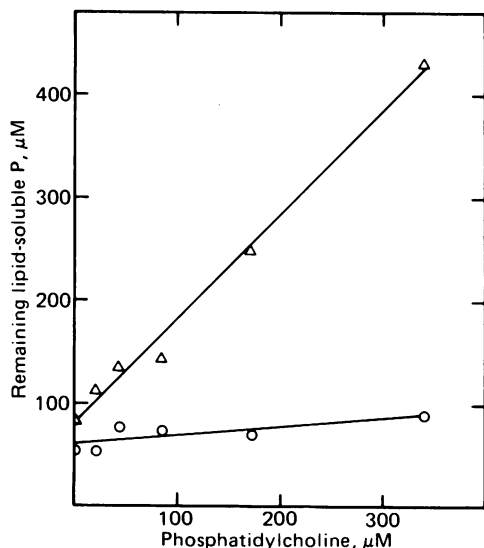


FIG. 1. Hydrolysis of exogenous phosphatidylcholine by phospholipase C. A solution of egg phosphatidylcholine in ethanol was placed in a sterile tube and evaporated to dryness with N₂. Culture medium (2 ml) was added and the mixture was briefly sonicated to disperse the phosphatidylcholine, the final concentration of which was 6.4 mM. Various amounts of this dispersion were added to the cultures on day 1 at the same time as the phospholipase C. After 24 hr the amount of remaining lipid-soluble phosphorus was determined. The cells were grown in the absence (Δ) or presence (○) of phospholipase C.

RESULTS

Activity of Phospholipase C in Myoblast Cultures. To determine whether phospholipase C is active under the culture conditions, increasing amounts of exogenous phosphatidylcholine were added to the culture medium and the amount remaining after 24 hr was determined. In the absence of phospholipase C the phosphatidylcholine remained stable, but in the presence of phospholipase C the phosphatidylcholine was extensively degraded (Fig. 1). Even at the highest concentration of phosphatidylcholine, which represented a 20-fold molar excess over the total cellular phosphatidylcholine, over 95% of the added phospholipid was degraded.

Stimulation of Phospholipid Synthesis in Phospholipase C-Treated Cells. Although the phospholipase C is active in the culture medium, access of the enzyme to plasma membrane phospholipids might be hindered by other cell surface components, such as glycoproteins and glycolipids. Therefore, it was necessary to determine the effect of phospholipase C on the content and metabolism of cellular phospholipids. Phospholipid synthesis was stimulated considerably in cells grown in the presence of phospholipase C (Fig. 2). After a brief lag, the rate of incorporation of ³²P_i into total phospholipids was 4- to 5-fold greater in phospholipase C-treated cells than in untreated cells. The rate of labeling of the trichloroacetic acid-soluble portion of the cell homogenate was essentially the same in control and phospholipase-treated cells, indicating that the rate of phosphate transport into the cells was not altered by the phospholipase C treatment. From the specific radioactivity of the ³²P_i in the medium and the average phospholipid content of the cells, the amount of phospholipid synthesized in 4.5 hr in the phospholipase C-treated cells represented at least 8% of the cellular phospholipid. This is a minimum value because the ³²P_i is diluted by cytoplasmic pools.

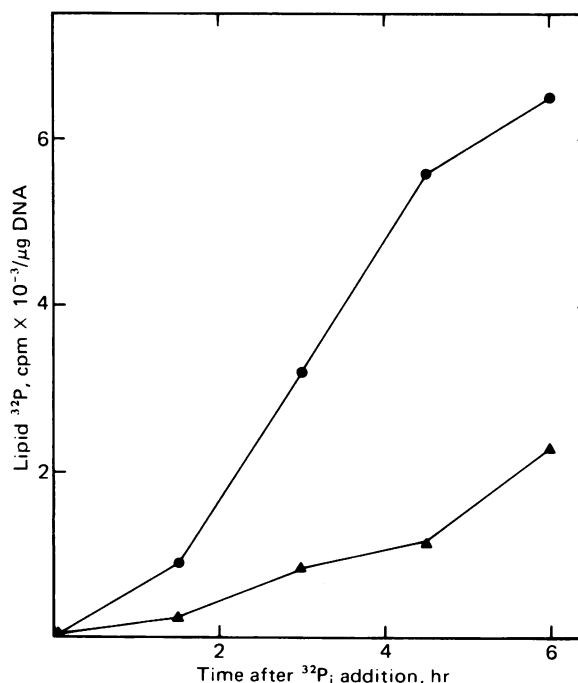


FIG. 2. Effect of phospholipase C on ³²P incorporation into phospholipids. On the third day of culture, ³²P_i (8 μ Ci/ml; 1 Ci = 3.7 \times 10¹⁰ becquerels) was added to cells that had been grown in the absence (▲) or presence (●) of phospholipase C. The cells were harvested at the indicated times and lipid extractions and DNA determinations were performed on the cell homogenate. To normalize for variations in cell density, the total cpm in lipid from each dish was divided by the total micrograms of DNA from each dish.

Table 1. Phospholipid biosynthesis in fusing and nonfusing muscle cells

Cells	Culture conditions	Lipid, cpm/ μ g of DNA	
		^3H	^{32}P
Fusing	High Ca^{2+} , -PLC	1510 \pm 170	800 \pm 120
Nonfusing	Low Ca^{2+} , -PLC	1630 \pm 30	630 \pm 10
Nonfusing	High Ca^{2+} , +PLC	9070 \pm 1700	4570 \pm 770

On the third day of culture, [^3H -methyl]choline and $^{32}\text{P}_i$ were added to final concentrations of 1 and 5 $\mu\text{Ci/ml}$, respectively. The cells were harvested 4 hr later. The numbers represent the mean \pm SD for duplicate dishes. PLC, phospholipase C.

In the experiment shown in Fig. 2 the control cells were fusing but the phospholipase C-treated cells were not. Possibly the higher rates of $^{32}\text{P}_i$ incorporation into lipids of phospholipase-treated cells would be observed in all nonfusing cells. The incorporation of radioactive precursors into lipids of phospholipase-treated cells was compared to that in cells inhibited from fusing by calcium deprivation (11) (Table 1). Incorporation of either [^3H -methyl]choline or $^{32}\text{P}_i$ into lipids was the same in the nonfusing, low-calcium cells as in the fusing, high-calcium cells (Table 1). Moreover, isotope incorporation was 5- to 6-fold higher in the phospholipase C-treated cells than in either the fusing or nonfusing, low-calcium cells. These data indicate that the stimulation of lipid synthesis observed in phospholipase C-treated cells is not a property common to all nonfusing cells.

Although there is an increase in the rate of incorporation of isotopes into phospholipids in phospholipase C-treated cells, the phospholipid content of the crude particulate fraction from treated cells was essentially the same as that of the untreated cells, 0.183 \pm 0.027 and 0.208 \pm 0.002 nmol of lipid P per mg of protein (mean \pm SD from three preparations). Levels of the phospholipid classes were also the same in the treated and untreated cells (Fig. 3 left). After a 5-hr incubation of the cells with $^{32}\text{P}_i$, however, the specific radioactivities of the various phospholipid classes were quite different (Fig. 3 right). The greatest differences were in the choline-containing lipids. The specific radioactivity of phosphatidylcholine, the major phospholipid of these cells, was 13-fold higher in the phospholipase-treated cells than in the control cells. The specific radioactivity of sphingomyelin was over 20-fold higher in the

phospholipase-treated cells. Phosphatidylethanolamine and phosphatidylserine were also somewhat more highly labeled in the phospholipase-treated cells, whereas there were no differences in specific radioactivities of phosphatidylinositol and cardiolipin between the treated and untreated cells.

Degradation of Phospholipids in Phospholipase-Treated Cells. Phospholipase C-mediated degradation of phospholipids of the myogenic cells was observed by measuring both depletion of phosphatidylcholine from the cells and appearance of phosphocholine in the culture medium. Phospholipase-treated cells were grown in the presence of $^{32}\text{P}_i$ for 2 days to label the cellular phospholipids and then were incubated in nonradioactive medium in either the presence or absence of phospholipase C. As expected, there was a greater loss of [^{32}P]-phosphatidylcholine from cells incubated with phospholipase C (Fig. 4).

At various time intervals after the addition of nonradioactive medium, the level of [^{32}P]phosphocholine in the medium was determined by ion-exchange chromatography. The release of [^{32}P]phosphocholine into the medium was greatly enhanced in the presence of phospholipase C (Fig. 5). After the first 4 hr, the rate of appearance of labeled phosphocholine in the medium roughly paralleled the decline in [^{32}P]phosphatidylcholine in the cells (Fig. 4). The apparent stability of the cellular phosphatidylcholine during the first 4 hr was probably due to continued incorporation of radioactive cytoplasmic precursors into lipid during that time. The only other ^{32}P -labeled compound that reproducibly appeared in the culture medium, as detected by ion-exchange chromatography, was inorganic phosphate.

Alkaline phosphatase is a constituent of the plasma membranes of some cells (22). The differences observed in phosphocholine (Fig. 5) may have resulted from a more active phosphatase in the cultures incubated in the absence of phospholipase C rather than from differences in the rates of phosphocholine production. This possibility was excluded by an experiment in which phospho[^3H -methyl]-choline was added to the cultures and the amount remaining was determined after 3.5 and 7 hr (Table 2). Although some phospho[^3H -methyl]-choline was hydrolyzed, the amount of degradation was the same in the presence and absence of phospholipase C.

Phosphocholine is both a product of phospholipase C and a biosynthetic precursor of phosphatidylcholine. The higher re-

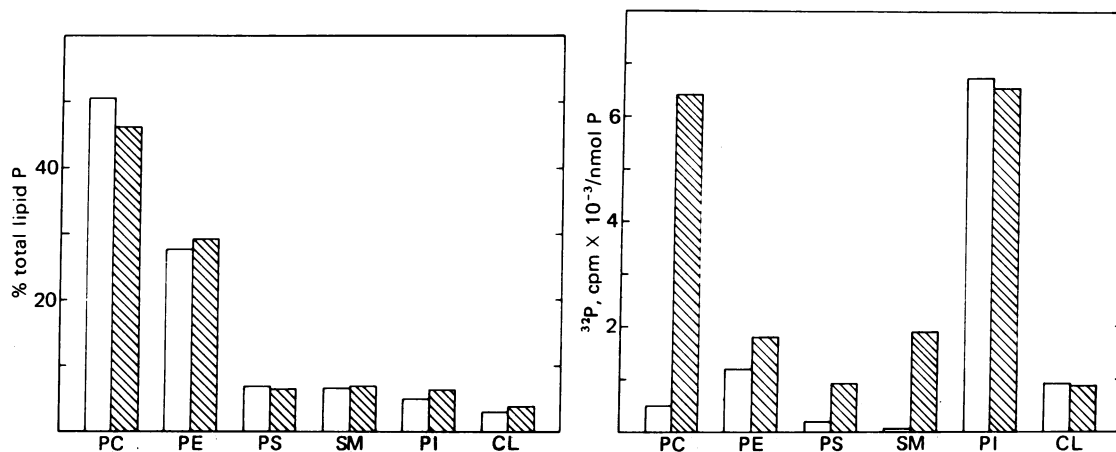


FIG. 3. Phospholipid composition of cells grown in the presence or absence of phospholipase C. Muscle cells were grown from day 1 in the absence (open bars) or presence (hatched bars) of phospholipase C. On the third day $^{32}\text{P}_i$ was added to the medium to a final concentration of 34 $\mu\text{Ci/ml}$ and the cells were harvested 5 hr later. Phospholipids were extracted from the crude particulate fraction and analyzed by two-dimensional chromatography. (Left) Percent of total lipid phosphate. (Right) Specific radioactivities of phospholipids from cells grown in the presence or absence of phospholipase C. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; PI, phosphatidylinositol; CL, cardiolipin.

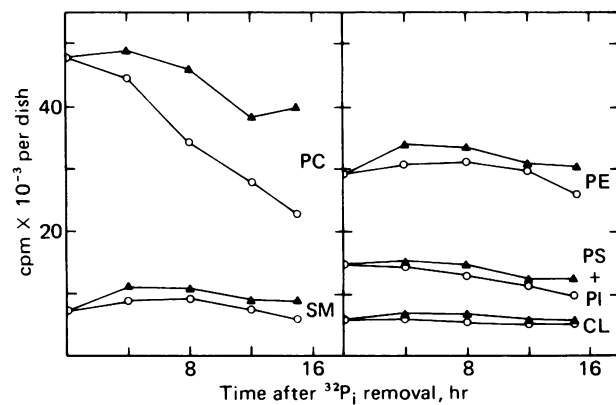


FIG. 4. Degradation of phospholipids in the presence and absence of phospholipase C. All cells for this experiment were grown in the presence of phospholipase C. $^{32}\text{P}_i$, 1.5 $\mu\text{Ci/ml}$, was present from the time of plating. On the third day of culture the radioactive medium was removed and the cells were washed three times in nonradioactive culture medium. The cells were incubated in nonradioactive culture medium in the presence or absence of phospholipase C and then were harvested at the indicated times. Lipids were extracted from the total homogenate and separated by one-dimensional chromatography. The data were normalized to the total radioactivity in cells plus medium at the time of harvest. ▲, Without phospholipase C; ○, with phospholipase C. See Fig. 3 legend for abbreviations.

lease of phosphocholine (Fig. 5) was possibly due to spillage of cytoplasmic phosphocholine as a result of cell lysis. However, cell lysis, as detected by release of the cytoplasmic marker lactate dehydrogenase into the culture medium, was similarly low (7% in 7 hr) in the presence or absence of the phospholipase. The high levels of radioactive phosphocholine appearing in the culture medium of phospholipase C-treated cells therefore resulted not from cell lysis but from enzymatic hydrolysis of cellular phospholipids.

DISCUSSION

The present studies were begun in an attempt to determine the mechanism by which phospholipase C inhibits membrane fusion in cultures of developing chicken muscle cells. The experiments reported in this paper were designed to determine whether phospholipase C affects phospholipid composition or metabolism in the myogenic cells. The activity of phospholipase C in muscle cell cultures was demonstrated by several experi-

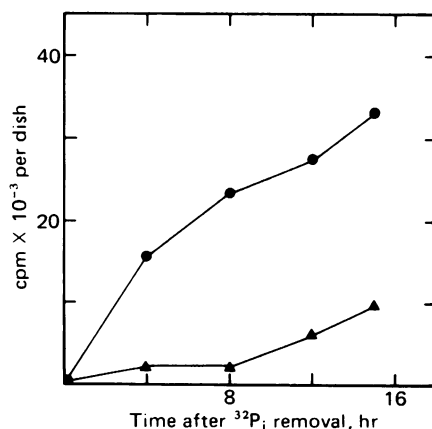


FIG. 5. Appearance of phosphocholine in the culture medium. These data were obtained from the same experiment as in Fig. 4. Phosphocholine was analyzed by ion exchange chromatography. ▲, Without phospholipase C; ●, with phospholipase C.

Table 2. Stability of phosphocholine in culture medium

Culture condition	Time of incubation, hr	cpm recovered as phosphocholine
-Phospholipase C	0	3300
	3.5	2900
	7.0	2290
+Phospholipase C	0	3300
	3.5	3105
	7.0	2354

On the third day of culture, phospho[methyl- ^{14}C]choline, 20,000 cpm/nmol, was added to a concentration of 1×10^5 cpm/ml of medium. At the indicated times the medium was removed and the phosphocholine content in a 50- μl sample was analyzed electrophoretically.

ments. The enzyme is able to degrade exogenous phosphatidylcholine added to the cultures, indicating that phospholipase C is active under the culture conditions. The enzyme also hydrolyzes myoblast phospholipids as demonstrated by increased degradation of radioactive cellular phosphatidylcholine accompanied by appearance of phosphocholine in the culture medium. Moreover, the biosynthesis of certain phospholipids is stimulated in cultures treated with phospholipase C, as determined by measuring incorporation of radioactive precursors into phospholipids. It should be noted that the actual rates of phospholipid synthesis have not been measured by this procedure. The rates of incorporation of the isotopic precursors reflect both differences in rates of phospholipid synthesis and differences in the sizes of cytoplasmic pools of precursors such as ATP, glycerol phosphate, phosphocholine, etc. The specific radioactivities of phosphatidylinositol and cardiolipin do not differ after a 5-hr incubation with $^{32}\text{P}_i$ (Fig. 3 right), suggesting that the specific radioactivities of the pools of ATP and glycerol phosphate, precursors of phosphatidylinositol and cardiolipin, are the same in control and phospholipase-treated cells. Preliminary evidence obtained after long-term labeling with $^{32}\text{P}_i$ and [^3H]choline indicates that cytoplasmic choline pools are also the same and CDP-choline is undetectable. The cytoplasmic phosphocholine pool, however, appears to be 2 to 3 times larger in cells grown in the absence of phospholipase C than in the presence of the enzyme. The 13-fold difference in specific radioactivities of phosphatidylcholine (Fig. 3 right) might, therefore, reflect only a 4- or 5-fold difference in rates of phosphatidylcholine synthesis.

The incorporation of radioactive precursors into sphingomyelin is greatly enhanced in phospholipase C-treated cells, but the rate of degradation of that lipid is not increased. The reason for this apparent discrepancy is not presently known. However, the kinetics of radiolabeling of sphingomyelin in these cells are consistent with synthesis of that compound directly from phosphatidylcholine (23, 24), the specific radioactivities of which differ considerably in phospholipase-treated and control cells.

The total phospholipid composition of cells treated with phospholipase C is not detectably different from that of the control cells, a fact consistent with acceleration of both synthesis and degradation. The phospholipid content of the plasma membranes may be significantly altered, but I have so far been unable to isolate purified plasma membranes from the phospholipase C-treated cells. The procedure used to purify plasma membranes from cultured muscle cells (2) is based on density gradient centrifugation of membrane vesicles. The plasma membrane markers from phospholipase C-treated cells are associated with fractions that are denser than those of the untreated cells, suggesting a lower lipid-to-protein ratio in the treated cells or altered permeability to water or sucrose.

The mechanism by which phospholipase C inhibits myoblast fusion is presently unknown. The inhibition may be related to the stimulation of lipid metabolism in these cells, but the two effects may be independent. In any event, it is clear that phospholipase C profoundly alters myoblast phospholipid metabolism, and so this system should prove extremely valuable in studying the regulation of membrane lipid synthesis. Indeed, the data presented in this paper suggest that phospholipid synthesis in myogenic cultures may be subject to regulation. It appears that the phospholipid synthetic apparatus is able to respond to the degradation of plasma membrane lipids by increasing the rates of synthesis of those lipids that are degraded by the phospholipase C. Questions as to which enzymatic step(s) is(are) regulated, what factors influence enzymatic activity, and how the information about lipid composition of the plasma membrane is transmitted to the endoplasmic reticulum, the usual site of phosphatidylcholine biosynthesis (25), remain to be answered.

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