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THE RELATIONSHIP BETWEEN CELL PROLIFERATION AND PHOSPHATIDYLINOSITOL METABOLISM IN SKELETAL MUSCLE CELLS IN CULTURE

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ABSTRACT

Effects of γ-hexachlorocyclohexane (1×10^{-4} M) and lithium chloride (10 mM) on the cell proliferation and phosphatidylinositol metabolism of L_6 myoblasts were examined. γ-Hexachlorocyclohexane (inhibitor of phosphatidylinositol synthesis) and LiCl (inhibitor of inositol 1-phosphatase) treatment of these cells resulted in the loss of their proliferative properties. Coordinate with effects on cell proliferation, γ-hexachlorocyclohexane and LiCl also reduced the levels of phosphatidylinositols and their water-soluble metabolites. However, the protein content of treated cells was higher than control cells. Gamma-hexachlorocyclohexane and LiCl treatment of cells reduced DNA synthesis by about 50% over control values. It was, therefore, concluded that there appears to be a direct relationship between L_6 myoblast proliferation and phosphatidylinositol metabolism.

(Key Words: Cell Culture, Cell Growth, Cell Division, Phosphatidylinositols.)

Introduction

The general outline of muscle growth has been extensively reviewed (Stromer et al., 1974; Allen et al., 1979). However, the mechanisms(s) responsible for control of myogenic cell proliferation have largely been ignored. Little is known about biochemical events responsible for triggering the proliferation process of myogenic cells. It is believed that there is little increase in muscle fiber number during postnatal development and that postnatal growth is primarily due to myofiber hypertrophy coupled with addition of nuclei to the existing muscle fibers during postnatal growth (Allen et al., 1979). Therefore, in order for any manipulation of embryonic cell proliferation to be successful, such manipulations must be accomplished while prenatal muscle cells are still proliferating and prior to differentiation. This is a critical point since it is believed that once myogenic cells have withdrawn from the mitotic cycle, they cannot re-enter the mitotic cycle (Yaross and Konigsberg, 1985).

Several lines of evidence indicate that calcium is involved in some way in the control of cell proliferation (Durham and Walton, 1982). The hydrolysis of phosphatidylinositol as a mechanism for generation of a secondary messenger for the release of intracellular calcium is an important and active area of research. Phosphatidylinositols are unique and minor constituents of eukaryotic cell membranes. They are unique because hormones and neurotransmitters that use calcium as a secondary messenger specifically hydrolyze phosphatidylinositol (for review, see Downes and Michell, 1985). The initial reaction is the receptor-mediated hydrolysis of phosphatidylinositol to give two products: diacylglycerol and inositol phosphates (figure 1). Among the

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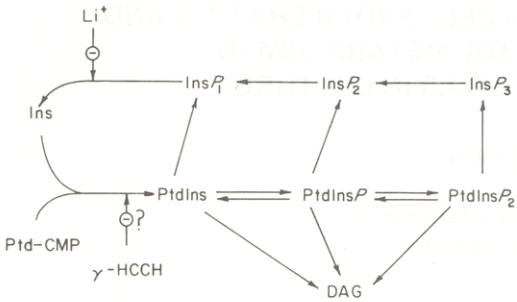

Figure 1. Biosynthesis and degradation of inositol lipids and inositol phosphates. Abbreviations used: Ptd (phosphatidyl), Ins (myoinositol), P (phosphate), DAG (1,2-diacylgylcerol), CMP (cytidine monophosphate) and γ-HCCH (γ-hexachlorocyclohexane).

species of inositol phosphates generated, inositol 1,4,5-triphosphate seems to act as a secondary messenger for the release of calcium (Streb et al., 1983; Burgess et al., 1984; Dawson and Irvine, 1984; Joseph et al., 1984; Prentki et al., 1984; Muallem et al., 1985). Because it has been shown that phosphatidylinositol hydrolysis results in the release of intracellular calcium and that calcium is involved in the control of cell proliferation, the objectives of studies described herein were to examine the relationship between phosphatidylinositol metabolism and cell proliferation of L_6 myoblasts.

Materials and Methods

Cell Culture Conditions. The L_6 myoblasts (originally obtained from Dr. Schubert at Salk Institute) were grown in Dulbecco's modified Eagle medium[4] supplemented with 10% fetal bovine serum (DMEM + 10% FSB). Cultures were grown in a humidified incubator at 37 C under 10% CO_2 atmosphere.

Cell Growth. Approximately 1×10^5 cells were plated in 35-mm, 6-well cluster dishes[5] in 2 ml of DMEM + 10% FBS and allowed to attach to the substrate. After attachment (12 h), medium was removed and 2 ml of fresh medium corresponding to the appropriate treatment was added. At this point, cell counts were obtained to ensure equal initial cell density. Twenty-four hours later, cells were refed with 2 ml of new medium corresponding to the treatment. Gamma-hexachlorocyclohexane[6] was dissolved in dimethylsulfoxide[6] (DMSO), and appropriate volumes were added to the media to give the final concentration of 1×10^{-4} M γ-hexachlorocyclohexane. The media was then sterilized by filtration (.2 μm[7]) and added to the dishes (control and treated media contained .035% DMSO). Seventy-two hours after original plating, medium was removed and cells were trypsinized (.05% trypsin in Ca^{++}, Mg^{++}-free Earle's salt solution) and counted in a ZBI Coulter Counter[8].

Pre-labelling of Cells with Myo-[2-^{3}H]inositol. At the same time that cells were plated for cell growth, parallel sets of 35-mm, 6-well cluster dishes were prepared by the initial plating procedure to be used for the prelabelling of cells with myo-[2-^{3}H] inositol. After attachment, the medium was removed and 2 ml of fresh medium, corresponding to appropriate treatment containing 2 μCi/ml of medium of myo-[2-^{3}H] inositol (specific activity 14 Ci/mmol, Amersham), was added. As before, cells were refed with fresh medium (containing myo-[2-^{3}H] inositol) 24 h after plating.

Extraction and Separation of Water-Soluble Inositol Metabolites. At appropriate times (as indicated in the text) plates were removed from the incubator and water-soluble inositol metabolites were extracted according to the following procedure. The medium containing labelled inositol was removed, and cells were rinsed twice and incubated for 1 h with 2 ml of phosphate-buffered saline (PBS), pH 7.4 (Berridge et al., 1984). After an additional rinse with PBS to remove as much free myo-[2-^{3}H]-inositol as possible, the reaction was stopped with 1 ml of ice-cold 15% trichloroacetic acid (TCA) and left on ice for 30 min. Cells were then harvested with rubber policemen and centrifuged at 2,000 × g for 5 min. This procedure was repeated two additional times with the addition of 1 ml of H_2O each time for maximum extraction of the water-soluble metabolites. The supernatants from the TCA extractions and H_2O washes were combined and TCA was removed by extraction with diethylether (four extractions). The final extraction was neutralized with NaOH and the ^{3}H content was determined for total water-soluble inositol metabolites, or applied to resin

[4] Gibco, Grand Island, NY.
[5] Costar, Cambridge, MA.
[6] Sigma Chemical Co., St. Louis, MO.
[7] Millipore Corp., Bedford, MA.
[8] Coulter Electronics, Inc., Hialeah, FL.

slurry (AGI-X8 in formate form[9]) to separate water-soluble metabolites (Berridge et al., 1982). The entire neutralized supernatant was incubated for 15 min with the resin slurry to allow for absorption of inositol phosphates. The slurry was then poured into small columns. The water-soluble metabolites were eluted by the stepwise addition of solutions containing increasing levels of ammonium formate (Berridge et al., 1982). [^{3}H] inositol was eluted with three × 2-ml washes with H_2O. Elution of bound water-soluble products was initiated by washing the columns with three × 2-ml aliquots of 200 mM ammonium formate + 100 mM formic acid (inositol monophosphate), 400 mM ammonium formate + 100 mM formic acid (inositol bisphosphate) and 1,000 mM ammonium phosphate + 100 mM formic acid (inositol trisphosphate). The ^{3}H content of samples was determined in liquid scintillation spectrophotometer.

Extraction of Phosphatidylinositols. Phosphatidylinositols were extracted from the trichloroacetic acid precipitates (see extraction of water-soluble inositol metabolites) in 1.5 ml of chloroform:methanol:12 N HCl (200:100:1); the lipids extracted were washed twice with 5-ml aliquots of .1 N HCl (Hokin-Neaverson and Sadeghian, 1984). For estimation of total lipid radioactivity, the chloroform phase was evaporated to dryness in a scintillation vial and ^{3}H content was determined.

^{3}H-Thymidine Incorporation. At the same time that cells were plated for cell growth, parallel sets of 35-mm, 6-well cluster dishes were prepared for measurement of ^{3}H-thymidine incorporation. Attachment medium was removed and fresh medium corresponding to the appropriate treatment was added. Twenty-four hours later, cells were refed with 2 ml of medium corresponding to the treatment containing .25 μCi/ml of medium of ^{3}H-thymidine (specific activity 20 Ci/mmol), and the cultures were incubated for 18 h. At the end of 18 h, the cultures were washed rapidly three times with cold PBS, and treated with 10% TCA for 10 min, followed by three rapid washes with 1% TCA. The plates were allowed to drain and the precipitated cellular materials were dissolved in .5 M NaOH at room temperature. To a minivial, .3 ml of this solution was added, acidified (to suppress fluorescence) with .1 ml of 5 M HCl, and radioactivity was determined in 4 ml of budget-solve[10].

^{3}H-Leucine Incorporation. For ^{3}H-leucine incorporation, L_6 myoblasts were allowed to attach and then new medium corresponding to appropriate treatment was added and plates were incubated for 72 h. After the incubation period, ^{3}H-leucine incorporation was determined by pulsing treated and control cultures for 2 h with 5 μCi/ml medium (2 ml) ^{3}H-leucine (specific activity 58.4 Ci/mmol). Cultures were processed and radioactivity was determined according to the procedure described under ^{3}H-thymidine incorporation.

Statistical Analysis. Data were analyzed by analysis of variance for a randomized complete block design; the significance of differences between means was determined by a Dunnett test.

Results and Discussion

The principal objective of these experiments was to examine the relationship between phosphatidylinositol and cell proliferation of L_6 myoblasts. An inhibitor of phosphatidylinositol biosynthesis (figure 1), γ-hexachlorocyclohexane (γ-HCCH), was first used by Hokin and Brown (1969). Since then, γ-HCCH has been used by a number of researchers to block the synthesis of phosphatidylinositol (Fisher and Mueller, 1971; Hoffmann et al., 1980; Vu et al., 1983; Zelenka and Vu, 1984; Koohmaraie and Schollmeyer, 1985; Koohmaraie et al., 1986). The specific site of action of γ-HCCH has not been established; researchers from one laboratory (Parries and Hokin-Neaverson, 1985) have suggested that γ-HCCH is not a selective inhibitor of phosphatidylinositol metabolism but that it has multiple effects. It is important to indicate, however, that these workers (Parries and Hokin-Neaverson, 1985) used 4mM γ-HCCH, a concentration about 40 times higher than those used by others. In the present study, 1.0 mM γ-HCCH was toxic to L_6 myoblasts because it resulted in detachment of these cells from the substrate (data not indicated). In addition, Hoffmann et al. (1980) reported that .3 mM γ-HCCH was near the toxic level for rat embryo fibroblasts. It seems reasonable to suggest that a number of effects observed by Parries and Hokin-Neaverson (1985) may result from the extremely high

[9] Bio-Rad Lab., Richmond, CA.

[10] Research Products International Corp., Mt. Prospect, IL.

concentration used. Furthermore, if these workers had used intact cells rather than partially purified enzymes, cell toxicity would be expected based on observation with other cell types. Because of concerns regarding γ-HCCH, it seemed necessary to examine the relationship between cell proliferation and phosphatidylinositol metabolism with another compound in addition to γ-HCCH. The chemical of choice was LiCl (10 mM) which has been shown to be a potent inhibitor of inositol 1-phosphatase (figure 1), an enzyme that catalyzes the conversion of inositol monophosphate to free inositol and inorganic phosphate (Hallcher and Sherman, 1980; Berridge et al., 1982; Best and Malaisse, 1983; Aub and Putney, 1984; Downes and Michell, 1985). Inhibition of inositol-1-phosphatase resulted in blockage of the resynthesis of phosphatidylinositol from free inositol (figure 1), the product of the enzyme (Hokin-Neaverson and Sadeghian, 1984). In the experiments on L_6 myoblasts, therefore, γ-HCCH was used to inhibit the synthesis of phosphatidylinositol and LiCl to inhibit the hydrolysis of phosphatidylinositol.

Table 1 indicates the effect of γ-HCCH and LiCl on cell proliferation, protein content, phosphatidylinositol and water-soluble metabolites content of the L_6 myoblasts. These results indicate that both γ-HCCH and LiCl reduced cell number, incorporation of ^{3}H-inositol into phosphatidylinositol and water-soluble metabolites of the L_6 myoblasts. However, the protein content of the treated cells was higher than in the control cells, suggesting that the protein synthesis apparatus was not adversely affected. To verify the effect of these compounds on cell proliferation and cell protein contents, ^{3}H-thymidine and ^{3}H-leucine incorporation was studied (table 2). Both γ-HCCH and LiCl inhibited incorporation of ^{3}H-thymidine or cell proliferation and stimulated ^{3}H-leucine incorporation or protein synthesis. This stimulation of ^{3}H-leucine incorporation could be due to the size of the treated cells rather than to actual stimulation of protein synthesis. Treated cells are much larger than control cells as judged by phase microscopy (figure 2; Koohmaraie et al., 1986).

Hoffmann et al. (1980) reported that .3 mM γ-HCCH was near toxic level for rat embryo fibroblasts and led to significant losses of cells in their experiments. To ensure that the observed effects of γ-HCCH and LiCl were not

TABLE 1. EFFECT OF γ-HEXACHLOROCYCLOHEXANE AND LITHIUM ON THE CELL PROLIFERATION AND PHOSPHATIDYLINOSITOL METABOLISM[a]

Item	Control	1×10^{-4} M γ-hexachlorocyclohexane	10 mM LiCl
Cell no. $\times 10^{-5}$	11.8 ± .14[c]	5.54 ± .14[d]	5.95 ± .14[d]
μg protein/10^5 cells	16.6 ± .36[c]	24.89 ± .36[d]	20.61 ± .36[e]
Phosphatidylinositols/μg protein, cpm[b]	81.2 ± 2.4[c]	50.8 ± 2.4[d]	54.3 ± 2.4[d]
Phosphatidylinositols/10^5 cells, cpm	1,357 ± 57[c]	1,280 ± 57[c]	1,234 ± 57[c]
Water-soluble metabolites/μg protein, cpm	221.0 ± 9.0[c]	147.8 ± 9.0[d]	177.8 ± 9.0[d]
Water-soluble metabolites/10^5 cells, cpm	3,610 ± 214[c]	3,700 ± 214[c]	3,667 ± 214[c]

[a]The values shown are mean ± pooled SE for six independent experiments with three observations in each experiment.

[b]cpm = Counts per minute.

[c,d,e]Means within a row with different superscripts differ ($P<.01$).

TABLE 2. EFFECT OF γ-HEXACHLOROCYCLOHEXANE AND LITHIUM CHLORIDE ON THE ^{3}H-THYMIDINE AND ^{3}H-LEUCINE INCORPORATION IN L_6 MYOBLASTS[a]

Treatment	Counts·min^{-1}·10^{-5} cells	
	^{3}H-thymidine	^{3}H-leucine
Control	42,782 ± 887[b]	4,638 ± 331[b]
1 × 10^{-4} M γ-hexachlorocyclohexane	22,028 ± 887[c]	5,975 ± 331[c]
10 mM LiCl	23,171 ± 887[c]	6,207 ± 331[c]

[a]The values shown are mean ± pooled SE for three independent experiments with three observations in each experiment.

[b,c]Means within a column with different superscripts differ (P<.01).

due to a toxic effect, cells were monitored via phase microscopy and photographed at the end of the experiments. Although cell proliferation and ^{3}H-thymidine incorporation were substantially attenuated by γ-HCCH and LiCl, the cells remained viable, did not become refractile and(or) released from the substrate with these treatments (figure 2). Other evidence for viability was the increased protein accumulation and ^{3}H-leucine incorporation in treated compared with control cells, and the observation that removal of γ-HCCH from non-fusing myoblasts allows resumption of proliferation (Koohmaraie et al., 1986). These results, therefore, demonstrate that while the L_6 myoblasts were apparently arrested, presumably in G_1 stage of cell cycle, with γ-HCCH and LiCl treatment, it seems very unlikely that the observed effects were due to a toxic effect of these agents.

The data in table 1 for incorporation of ^{3}H-inositol into phosphatidylinositol and water-soluble metabolites were expressed as cpm/μg of protein. The reason for expression of data in this manner was to take into account the effect of these treatments on the size of the treated cells. Microscopic inspection of treated and control cells and also data regarding protein/10^5 cells clearly demonstrated that treated cells were substantially larger than control cells (table 1, figure 2). This is an important point because when the data are expressed on a per-cell basis (10^5 cells), the values are virtually identical (table 1). To verify this point and to avoid questions regarding cell number and cell size, two additional experiments were carried out (tables 3 and 4). These additional experiments were designed to examine the effect of γ-HCCH and LiCl on phosphatidylinositol synthesis and hydrolysis at equal cell density.

To examine the effect of γ-HCCH and LiCl on phosphatidylinositol synthesis at equal cell density, growth-arrested cells were pre-incubated with γ-HCCH or LiCl for 75 min; incubation then continued for an additional 30 min in the presence of ^{3}H-inositol (see table 3 for details). These results clearly show that γ-HCCH inhibited phosphatidylinositol synthesis by 71% over control cells, whereas LiCl had no effect on the synthesis of phosphatidylinositol. This result (i.e., inhibition of phosphatidylinositol synthesis by γ-HCCH) is in agreement with results obtained with other cell types (Hoffmann et al., 1980; Vu et al., 1983; Zelenka and Vu, 1984).

To examine the effect of γ-HCCH and LiCl on the hydrolysis of phosphatidylinositol, growth-arrested cells were pre-labelled with ^{3}H-inositol for 120 min and then incubated with γ-HCCH and LiCl in the absence of the ^{3}H-inositol (see table 4 for details). Results clearly demonstrate that LiCl inhibited the activity of inositol-1-phosphatase (figure 1) as evidenced by: 1) greater radioactivity in phosphatidylinositol, inositol monophosphate, inositol bisphosphate and inositol trisphosphate and 2) lower radioactivity of free inositol (table 4). The fact that LiCl-treated cells have accumulated phosphatidylinositol and inositol phasphates indicates that inhibition of inositol-1-phosphatase probably prevented the resynthesis of phosphatidylinositol. This effect of LiCl was reported by Hokin-Neaverson and Sadeghian (1984). Results of this experiment (i.e., inhibition of inositol-1-phosphatase by LiCl) are in agreement with those reported for other cell types (Hallcher and Sherman, 1980; Berridge et al., 1982; Best and Malaisse, 1983;

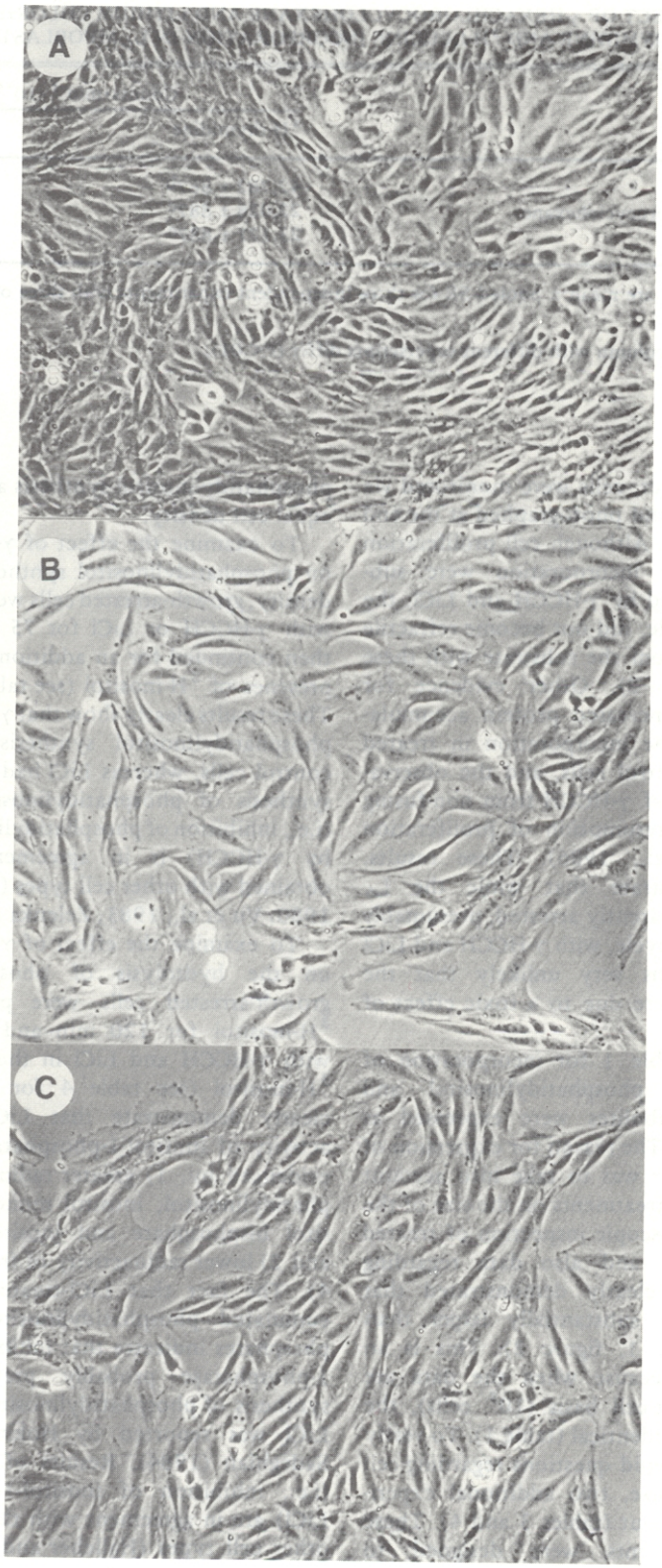
A
B
C

TABLE 3. EFFECT OF γ-HEXACHLOROCYCLOHEXANE AND LITHIUM CHLORIDE ON THE PHOSPHATIDYLINOSITOL SYNTHESIS[a]

Treatment	Count·min^{-1}·well^{-1}
Control	1,557 ± 151[b]
1 × 10^{-4} M γ-hexachlorocyclohexane	442 ± 151[c]
10 mM LiCl	1,478 ± 151[b]

[a]1 × 10^{-5} cells were plated for 48 h in DMEM + 10% FBS and then 12 h in DMEM + 1.5% FBS (to allow cells to complete their cell cycle). At the end of 12 h, cell counts were obtained (three out of six wells). Growth-arrested cells were then incubated with DMEM alone, 1 × 10^{-4} M γ-hexachlorocyclohexane in DMEM and 10mM LiCl in DMEM for 75 min. After 75 min, 10% FBS was added together with 10 μCi/well of myo-[2-^{3}H] inositol for another 30 min. The reaction was then stopped and phosphatidylinositols were extracted as described in Materials and Methods. Results are expressed as count·min^{-1}·well^{-1}. The values shown are mean ± pooled SE for three independent experiments with three observations in each experiment.

[b,c]Means within a column with different superscripts differ ($P<.01$).

Aub and Putney, 1984; Hokin-Neaverson and Sadeghian, 1984).

Treatment of pre-labelled cells with γ-HCCH resulted in a significant decrease in radioactivity in phosphatidylinositol, accompanied by a significant increase in radioactivity in inositol monophosphate. Because this increase was only in inositol monophosphate and not in inositol bisphosphate or inositol trisphosphate, it can be concluded that γ-HCCH had a stimulatory effect on the breakdown of phosphatidylinositol only, and not on the degradation of phosphatidylinositol 4-phosphate or phosphatidylinositol 4,5-biphosphate (figure 1). Because the hydrolysis of phosphatidylinositol 4,5-bisphosphate generates inositol trisphosphate and because inositol trisphosphate is the secondary messenger for the release of intracellular Ca^{++} (Streb et al., 1983; Burgess et al., 1984; Dawson and Irvine, 1984; Joseph et al., 1984; Prentki et al., 1984; Muallem et al., 1985), γ-HCCH would not be expected to stimulate cell proliferation caused by release of intracellular Ca^{++}.

The data presented here clearly document a profound negative effect of γ-hexachlorocyclohexane and LiCl on the proliferative abilities of L_6 myoblasts. These results, when considered collectively with similar results obtained with other cell types (Hoffmann et al., 1980; Zelenka and Vu, 1984; Carney et al., 1985; Koohmaraie and Schollmeyer, 1985; Koohmaraie et al., 1986), lend support to the hypothesis that cell proliferation and phosphatidylinositol metabolism are linked. The precise manner in which these two events are interrelated is not yet understood. Phosphatidylinositol could affect the cell proliferation process by generating inositol phosphates and(or) diacylglycerol. Inositol phosphates could affect the cell proliferation process by mobilizing intracellular Ca^{++}, while diacylglycerol could affect this process by activation of the Na^+/H^+ exchange system (Rindler et al., 1979; Moolenaar et al., 1982; Vigne et al., 1982, 1985; Frelin et al., 1983). The results of the experiment reported here and those of Vigne et al. (1985), who reported that the Na^+/H^+ exchange system could be activated in proliferative myoblasts and not in differentiated myotubes, dictates that additional work should be directed toward understanding the interrelationship between cell proliferation and phosphatidylinositol metabolisms.

Figure 2. Phase-contrast micrograph of L_6 myoblasts grown in the: a) control media; b) control media containing 1 × 10^{-4} M γ-hexachlorocyclohexane and c) control media containing 10 mM LiCl for 72 h.

TABLE 4. EFFECT OF γ-HEXACHLOROCYCLOHEXANE AND LITHIUM CHLORIDE ON THE PHOSPHATIDYLINOSITOL HYDROLYSIS[a]

Treatment	Phosphatidylinositols	Inositol	Inositol monophosphate	Inositol bisphosphate	Inositol trisphosphate
Control	14,790 ± 805[b]	24,733 ± 1,297[b]	751 ± 262[e]	107 ± 10[e]	156 ± 15[e]
1×10^{-4} M γ-hexachlorocyclohexane	12,498 ± 805[c]	28,264 ± 1,297[b]	2,004 ± 262[f]	106 ± 10[e]	137 ± 15[e]
10 mM LiCl	17,198 ± 805[d]	18,248 ± 1,297[c]	4,958 ± 262[g]	258 ± 10[f]	336 ± 15[f]

[a] 1×10^{-5} cells were plated for 36 h in DMEM + 10% FBS and then 12 h in DMEM + 1.5% FBS (to allow cells to complete their cell cycle). At the end of the 12 h, cell counts were obtained (three out of six wells). Growth-arrested cells were then pre-labelled 120 min with myo-[2-^3H] inositol. Cells were then rinsed four times with 2-ml aliquots of DMEM alone and then incubated with appropriate treatments for 30 min. The reaction was stopped and phosphatidylinositols and water-soluble metabolites were extracted as described in Materials and Methods. Results are expressed as count•min^{-1}•well^{-1}. The values shown are mean ± pooled SE for three independent experiments with three observations in each experiment.

[b,c,d] Means within a column with different superscripts differ ($P<.05$).

[e,f,g] Means within a column with different superscripts differ ($P<.01$).

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