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PHOSPHOLIPID METHYLATION IN MYOGENIC CELLS

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The biosynthesis of phosphatidylcholine from successive N-methylation of phosphatidylethanolamine has been implicated as a major mechanism for the transduction of several receptor-mediated signals including β-adrenergic coupling to adenylate cyclase. In this report we demonstrate L-isoproterenol stimulation of adenylate cyclase activity in two myogenic cell lines, L8 and BC3H-1. Using a sensitive high performance liquid chromatography method for qualitative and quantitative determination of phospholipids we found an active membrane phospholipid methylation pathway in these cells. Despite β-adrenergic simulation of adenylate cyclase no alteration in the transmethylation pathway could be demonstrated.

Increases in sequential methylation of membrane phospholipids have been implicated in mediating the response of adenylate cyclase to β-adrenergic receptor activation (1,2). Also, stimulation of phospholipid methylation is thought to play a role in coupling other receptor mediated events such as mitogen stimulation of lymphocytes (3), antigen-stimulated histamine release (4,5), cellular response to chemoattractants (6,7), and differentiation of embryonic lens fiber cells (8). It might be anticipated, therefore, that membrane phospholipid methylation would mediate β-adrenergic stimulation of adenylate cyclase in muscle.

Abbreviations: EBSS, Earles balanced salt solution; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; TCA, trichloracetic acid; HPLC, high performance liquid chromatography; RIA, radio-immuno assay; PME, phosphatidyl-N-monomethylethanolamine; PDE, phosphatidyl-N-dimethylethanolamine; PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

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Muscle cell lines grown in culture have been useful for studying membrane properties of muscle as well as receptor mediated events. L8 is a myogenic cell line which fuses to form multinucleate myotubes (9). This cell line has well defined β-adrenergic receptors (10,11) which are coupled to adenylate cyclase; addition of β-adrenergic agonists causes an increase in the intracellular levels of cyclic AMP (11). There is no information available about the existence or role of phospholipid methylation in these cells.

BC3H-1 is a muscle cell line which does not fuse in culture (12). This non-fusing cell line, however, exhibits many of the properties of muscle including electrical excitability (12), measurable amounts of adenylate kinase and creatine phosphokinase (12), synthesis of acetylcholine (13), and α-adrenergic receptors (14). There is similarly no information available about the presence or possible coupling role of phospholipid methylation in this myogenic cell line.

In this report, we demonstrate that L-isoproterenol added to both fusing and non-fusing myogenic cell lines stimulate adenylate cyclase activity. We also show that both cell lines have an active membrane phospholipid methylation pathway. However, L-isoproterenol stimulation of cyclic AMP synthesis is not coupled to the phospholipid methylation pathway. Despite increases in cyclic AMP levels by as much as 40 fold after stimulation, phospholipid methylation was unaffected in either of the myogenic cell lines. These results raise serious doubts about the role of phospholipid methylation in mediating β-adrenergic receptor-stimulated adenylate cyclase activity.

MATERIALS AND METHODS

Materials. L-[methyl-^{3}H]-methionine (15ci/mmole) was obtained from Amersham Searle (Arlington Heights, Illinois) aliquoted and stored light-free under nitrogen. Cyclic AMP RIA kits were

obtained from Collaborative Research Inc. (Waltham, Massachusetts). Ficoll-Paque was obtained from Pharmacia Fine Chemicals (Piscataway, New Jersey). N-hexane and propanol-2 were obtained from Burdick and Jackson Laboratories, Inc. (Muskegon, Michigan); phospholipid standards were purchased from Cal Biochem (La Jolla, California). Radioactive standards were purchased from Amersham Searle (Arlington Heights, Illinois), all other chemicals were obtained from Sigma (St. Louis, Missouri).

Cell culture. The clonal muscle cell lines BC3H-1 and L8 were grown in Dulbecco-modified Eagle's medium containing 10% fetal calf serum, 100 units penicillin and 100mcg/ml streptomycin (growth medium) in a 12% carbon dioxide, 88% air, 37°C incubator. They were maintained in the logarithmic phase of growth by passage every 4-5 days. Log phase cells were removed from the dish with trypsin (Sigma). Cell number was determined by visual counts with a hemocytometer. Cells were plated in 9.62 cm^2 plastic tissue culture dishes at 3.4 x 10^4 cells/cm^2. BC3H-1 was grown in culture dishes which were collagen coated. Cells were allowed to enter the stationary phase of growth and used on day 7 or 8.

Phospholipid Methylation. Plated confluent cells were washed of their media with Earles balanced salt solution containing 25mm HEPES at pH 7.4. Cells were then incubated at 37°C for 60 minutes with L-[methyl ^{3}H]-methionine in EBSS/HEPES to label the S-adenosyl-L-methionine pool and subsequently washed with EBSS/HEPES. The reaction was started by the addition of 0.1mM L-isoproterenol and the cells were incubated for either 5, 10, or 15 minutes. The reaction was performed with and without 10mM theophylline. There was no alteration of methylation activity by theophylline. The reaction was stopped by the addition of cold 10% trichloroacetic acid. Cells were placed on ice for 10 minutes and then washed with cold 10% TCA. This was removed and cells were air dried for 5 minutes.

Phospholipids were extracted from the TCA precipitate by 2 ml of n-hexane: propanol-2 (3:2 vol:vol)(15,16). The cells were gently rotated with the extracting solvents for 10 minutes. The n-hexane:propanol-2 was removed and saved. This process was repeated twice for a total extraction volume of 6 ml and then the extracting solvents were dried under nitrogen. The residual was resuspended in 0.5 ml of n-hexane:propanol-2:water (6:8:0.75, vol:vol:vol) and stored under nitrogen at 0°C. Fifty microliters of this was directly counted on a scintillation counter to obtain total [^{3}H-methyl] incorporation into the lipid extracted pool.

Phospholipid Separation. Separation and quantitation of phospholipids were achieved by injecting 200 microliters of the extracted sample into a Varian 5000 high performance liquid chromatograph (Varian, Palo Alto, California) equipped with a Micropak Si-5 silica gel column. The solvent system consisted of two reservoirs, one containing hexane:isopropanol (3:4, solvent A) and the second containing 89.5% solvent A and 10.5% water (solvent B). Phospholipid elution was accomplished with a gradient from 55% solvent A and 45% solvent B to 1% solvent A and 99% solvent B over the first ten minutes. This was held constant for fifteen minutes then returned to 55% solvent A and 45% solvent B for a remaining fifteen minutes. Flow rate was 1.0ml/min. This HPLC method was modified from that described by Geurts Van Kessel et al.(17) and James et al.(16). The eluate was monitored for radioactivity using a Radiomatic Flo-one Model HP radioactive flow through detector (Radiomatic, Tampa,

Florida). Identification of specific lipids was made by comparing their retention times to that of known standards purchased commercially and by performing thin layer chromatography on the peak fractions.

Other Determinations. Adenylate cyclase activity was determined using the RIA protocol devised by Collaborative Research Inc. (Waltham, Massachusetts). To stop the experimental reaction Ø.1 N HCl was substituted for 1Ø% TCA when simultaneous cAMP determinations were being performed. Proteins were determined by the method of Lowry et al (18) with bovine serum albumin as a reference protein.

RESULTS

A 4 to 4Ø-fold increase in catecholamine-stimulated endogenous cyclic AMP synthesis occurred between five and fifteen minutes after stimulation, in BC3H-1 and L8 respectively (Figure 1). In order to determine the extent to which phospholipid methylation was involved in the coupling of β-adrenergic stimulation to adenylate cyclase, simultaneous determinations of intracellular cyclic AMP and phospholipid methylation were performed (Figure 1). Despite the rapid and sustained stimulation of cyclic AMP, neither cell line showed any significant change in total [^{3}H-methyl] incorporation into extracted phospholipids. Since small

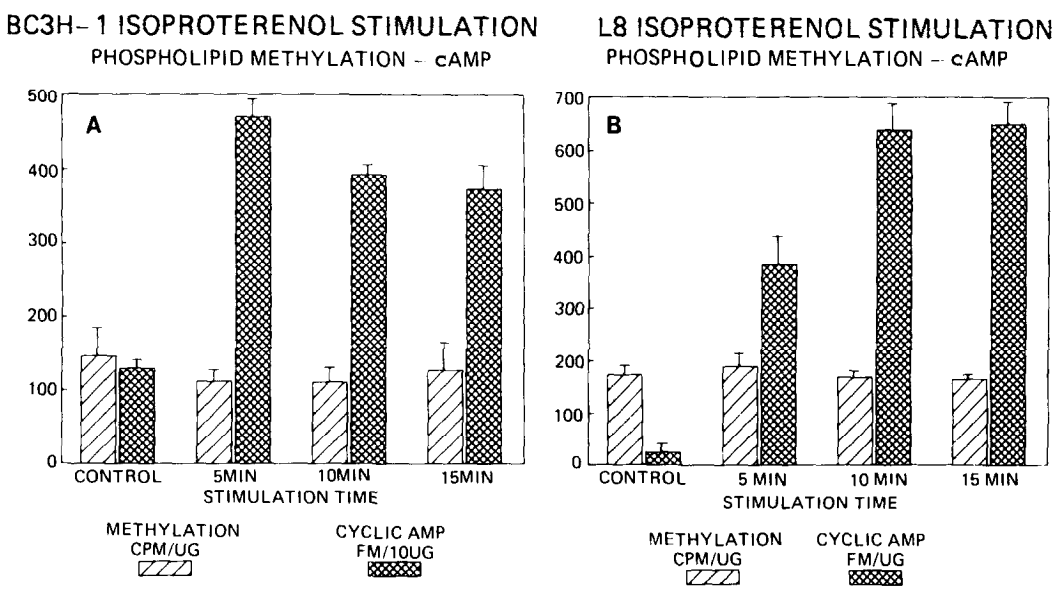

Figure 1. Phospholipid methylation and cyclic AMP accumulation in the presence of 1Ø mM theophylline as a function of time of stimulation with L-isoproterenol for BC3H-1 (A) and L8 (B). Assays as described in Methods.

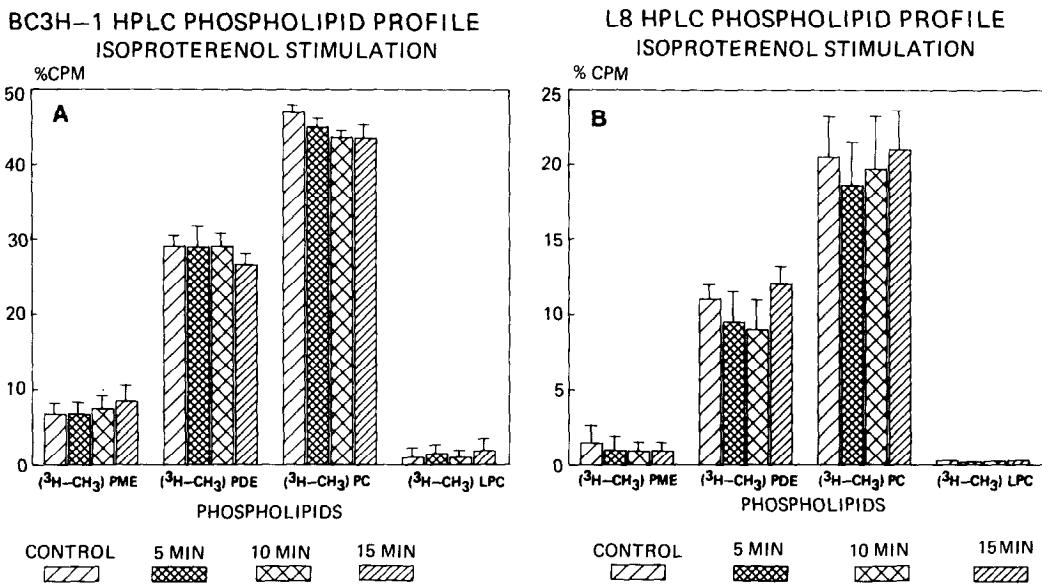

Figure 2. High performance liquid chromatography analysis of the phospholipids from BC3H-1 (A) and L8 (B) after stimulation with L-isoproterenol.

changes in the individual methylated phospholipid components may not be reflected by measuring total radioactivity, we used HPLC to separate and quantitate each component of the methyltransferase pathway. The order of elution of the lipids with retention times was: 1)phosphatidyl-N-monomethylethanolamine (14 minutes), 2)phosphatidyl-N-dimethylethanolamine (16 minutes), 3)phosphatidylcholine (18 minutes) and 4)lysophosphatidylcholine (22 minutes). This was established using both radiolabeled phospholipids and UV absorption of commercially obtained standard phospholipids. This was in agreement with previous reports (15).

The extracted lipids for each time point were then subjected to HPLC analysis. Each methylated component was examined for evidence of a catecholamine stimulated response (Figure 2). There was no observed alteration in any of the phospholipid components at any time point in either cell line. In addition to the N-methylated phospholipid components separated from the extracted membranes, a fifth radiolabeled fraction was

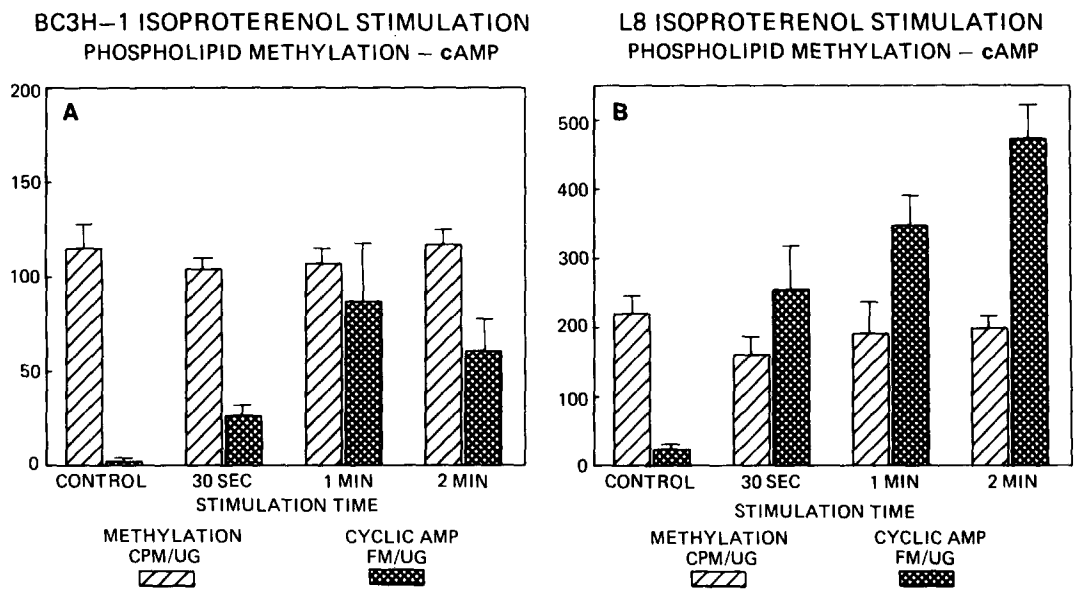

Figure 3. Phospholipid methylation and cyclic AMP accumulation in the presence of 10 mM theophylline as a function of time of stimulation with L-isoproterenol for BC3H-1 (A) and L8 (B). Assay as described in Methods.

consistently present. This was an early peak (2-3 minutes) which is probably methylated neutral lipids and glycolipids (15).

In order to exclude any possible early changes in phospholipid methylation in response to L-isoproterenol stimulation, time points at thirty seconds, one and two minutes were examined. In neither cell line was there any significant change in total [^{3}H-methyl] incorporation despite dramatic increases in cyclic AMP production (Figure 3). When HPLC was performed on these samples there was no alteration in any of the phospholipid components.

DISCUSSION

Both fusing and non-fusing myogenic cell lines respond to L-isoproterenol with increased adenylate cyclase activity and accumulation of intracellular cyclic AMP; cyclic AMP levels increased by as much as 40 fold in L8 cells. Since phospholipid methylation is reported to be coupled to receptor activation of adenylate cyclase (1,2) we searched for and found methyltransferase activities in these muscle cell lines.

Phospholipid methylation is characterized by the sequential N-methylation of phosphatidylethanolomine to phosphatidyl-N-monomethylethanolamine, phosphatidyl-N-dimethylethanolamine, and phosphatidylcholine by two methyltransferases in the cell membrane. S-adenosyl-L-methionine is the methyl donor for the reactions and methylation can be followed with radioactive labelling. Both fusing and non-fusing myogenic cells had comparable phospholipid methylation pathways. Although phospholipid methylation is only a minor source for phosphatidylcholine production (19), Hirata et al.(2) propose that enhanced phospholipid methylation produces local changes in membrane fluidity in response to cell membrane receptor activation. It is argued that these changes in membrane fluidity mediate membrane signal transduction by facilitating lateral mobility of the receptor complex and allowing interaction with coupling factors (2). We therefore searched for L-isoproterenol induced changes in phospholipid methyltransferase activity.

In most previously published reports, thin layer chromotography has been used to confirm the presence of the transmethylation pathway but radioactivity extractable into chloroform-methanol without chromatography is usually used to follow agonist stimulation of phospholipid methylation. Because this is not an adequate assay for measuring methylation, we used high performance liquid chromatography to resolve and quantitate individual products of the phospholipid methylation pathway before and after receptor activation. Despite marked activation of adenylate cyclase, we were not able to demonstrate any alteration in the methylation pathway.

These findings agree with recently reported studies using lymphocytes (20) and hepatocytes (21,22) to demonstrate a lack of phospholipid methylation coupling. Moore et al.(20) carefully

documented stimulation of phosphatidylinositol metabolism and [^{3}H] thymidine uptake with mitogenic stimulation of lymphocytes. However, they were unable to find any corresponding effect of the mitogens on phospholipid methylation. Schanche et al.(21) found that stimulation of adenylate cyclase activity in rat hepatocytes by β-adrenergic agents and glucagon was unaltered despite almost complete inhibition of the phospholipid methylation pathway. Colard and Breton (22) were unable to demonstrate any alteration in phospholipid methylation when rat liver plasma membrane adenylate cyclase was stimulated with glucagon. Recently Padel et al.(23) have shown that phospholipid methylation does not play a role in β-adrenergic-cyclic AMP mediated amylase secretion in the rat parotid gland. Taken together, these reports show that early changes in phospholipid methylation are not necessary for receptor coupling to adenylate cyclase. This indicates that careful measurement of methylated phospholipid substrates is absolutely necessary before concluding that activation of this pathway plays a role in receptor-mediated responses.

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REFERENCES

1. Hirata, F., Strittmatter, W., and Axelrod, J. (1979) Proc. Natl. Acad. Sci. USA 76, 368-372.
2. Hirata, F., and Axelrod, J. (1980) Science, 209, 1082-1090.
3. Hirata, F., Toyoshima, S., Axelrod, J., and Waxdel, M. (1980) Proc. Natl. Acad. Sci. USA 77, 862-865.
4. Hirata, F., Axelrod, J., and Crews, F. (1979) Proc. Natl. Acad. Sci. USA 76, 4813-4816.
5. Ishizaka, T., Hirata, F., Sterk, A., Ishizaka, K., and Axelrod J. (1981) Proc. Natl. Acad. Sci. USA 78, 6812-6818.

6. Hirata, F., Corcoran, B., Venkatasubramanian, K., Shiffmann, E. and Axelrod, J. (1979) Proc. Natl. Acad. Sci. USA 76, 2640-2643.
7. Pike, M., Kredich, N., and Snyderman, R. (1979) Proc. Natl. Acad. Sci. USA 76, 2922-2926.
8. Zelenka, P. S., Beebe, D. C., and Feagans, D. F. (1982) Science, 217, 1265-1267.
9. Yaffe, D. (1968) Proc. Natl. Acad. Sci. USA 61, 477-481.
10. Pochet, R., and Schmitt, H. (1979) Nature, 277, 58-59.
11. Schonberg, M., Bilezikian, J., Apfelbaum, M., and Benn, R. (1978) J. Cyclic Nucleot. Res. 4, 55-69.
12. Schubert, D., Harris, A., Devine, C., and Heinemann, S. (1978) J. Cell Biol. 61, 398-413.
13. Patrick, J., McMillan, J., Wolfson, H., and O'Brien, J. (1977) J. Biol. Chem. 252, 2143-2153.
14. Mauger, J. P., Sladeczek, F., and Bockaert, J. (1982) J. Biol. Chem. 257, 875-879.
15. Hara, A., and Radin, N. (1978) Analytical Biochemistry, 90, 420-426.
16. James, J. L., Clawson, G. A., Chan, C. H., and Smuckler, E. A. (1981) Lipids, 16, 541-545.
17. Geurts Van Kessel, W. S. M., Hax, W. M. A., Demel, R. A., and De Gier, J (1977) Biochimica et Biophysica Acta, 486, 524-530.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275.
19. Fischer, D. B., and Mueller, G. C. (1969) Biochim. Biophys, Acta. 176, 316-323.
20. Moore, J. P., Smith, G. A., Hesketh, T. R., and Metcalfe, J. C. (1982) J. Biol. Chem. 257, 8183-8189.
21. Schanche, J. S., Ogreid, D., Doskeland, S. O., Refsnes, M., Sand, T. E., Ueland, P. M., and Christoffersen, T. (1982) Febs Letters, 138, 167-172.
22. Colard, O., and Breton, M. (1981)Biochem. Biophys. Res. Commun. 101, 727-733.
23. Padel, U., Unger, C., and Soling, H-D. (1982) Biochem. J. 208, 205-210.