MODIFICATION OF GLYCEROLIPID METABOLISM IN L-M FIBROBLASTS BY AN UNNATURAL AMINO-ALCOHOL, N-ISOPROPYLETHANOLAMINE

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SUMMARY

The unnatural amino-alcohol, N-isopropylethanolamine, is incorporated into a phospholipid by monolayers of L-M fibroblasts. This phospholipid was identified as 1,2-diacyl-sn-glycero-3-phosphoisopropylethanolamine by using chemical and enzymatic procedures combined with thin-layer and gas-liquid chromatography. Since the phospho-N-isopropylethanolamine moiety is removed by phospholipase C, the stereochemistry of the phospholipid analog is identical to naturally occurring phosphoglycerides. Incubation of cells in 10 mM N-isopropylethanolamine inhibited the incorporation of [^{14}C]choline and [^{14}C]ethanolamine into phospholipids and stimulated the incorporation of [1-^{14}C]palmitic acid and [1-^{14}C]hexadecanol into triacylglycerols and alkyldiacylglycerols. These results indicate that N-isopropylethanolamine affects glycerolipid synthesis at the diradylglycerol branch point.

INTRODUCTION

Modification of membranes by using precursor analogs of the aliphatic or base portions of phospholipids has the potential of being exceedingly useful in assessing how lipids affect the structural and functional properties of membranes. Earlier studies in whole animals (1-8) and cell-free systems (9-12) have demonstrated that various substituted amino-alcohols, which do not occur in nature, can be incorporated into phospholipids of mammalian and insect tissues.

The work reported here was initiated to determine whether an unnatural amino-alcohol analog, IPE[1], could be incorporated into the phospholipids of L-M cells and to determine its effect on glycerolipid metabolism. Tissue cultures of L-M cells (13-15) were chosen for our studies, not only because they can be grown in a chemically defined media containing no serum, but because it has been shown that the acyl composition of their lipids can be altered by incorporation of fatty acids from the media (16,17). During the course of our investigation, Glaser and co-workers (18) reported that L-M cells could incorporate different amino-alcohol analogs into phospholipid molecules. However, the effect of such analogs on glycerolipid biosynthesis has not been determined.

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[1] IPE: N-isopropylethanolamine

MATERIALS AND METHODS

The L-M cell line (13), serum-free 199 media (14,15), and methods involved in feeding and transfer of the cell monolayers have been previously described (19). In each experimental series, the same number of cells were transferred in normal media and allowed to grow as monolayers to mid-log phase before adding the IPE. The monolayers were scraped loose and suspended in fresh media; relative cell numbers in the suspensions were determined by optical density measurements. Before extracting the cellular lipids (20), the media was removed and the monolayers gently washed twice with normal saline.

[1,2-^{14}C]Ethanolamine·HCl (6.3 μCi/μmole), [1,2-^{14}C]choline chloride (6.25 μCi/μmole), and [1-^{14}C]palmitic acid (56 μCi/μmole) were purchased from New England Nuclear. We prepared [1-^{14}C]hexadecanol from the [1-^{14}C]palmitic acid by reduction with Vitride (21). The labeled compounds were added to the media in 25 μl of ethanol and the monolayers incubated for 4 hr at 37°C.

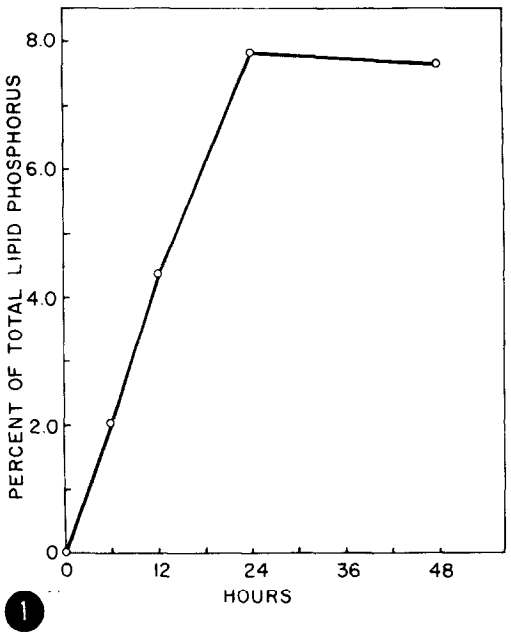
The IPE was prepared from acetone and ethanolamine essentially according to the procedure of Morrow et al. (22). The same technique was used to prepare ^{14}C-labeled IPE from [1,2-^{14}C]ethanolamine (2.0 μCi/μmole). Both preparations had purities of >98% as determined by TLC[1]. The IPE (10 mM) was dissolved in the media and the pH adjusted to 7.1-7.2 before adding to the monolayer flasks.

Neutral lipids were separated on thin-layer chromatoplates coated with Silica Gel G and developed in hexane:diethyl ether:acetic acid (80:20:1, v/v) or hexane:diethyl ether (90:10, v/v). Diacylglycerols were separated on the same plates using diethyl ether:hexane (60:40, v/v). Products obtained after Vitride reductions were chromatographed in diethyl ether saturated with water. Phospholipids were analyzed on Silica Gel HR layers developed in chloroform: methanol:acetic acid (50:25:8, v/v), chloroform:methanol:acetic acid:water (50:25:8:4, v/v), or chloroform:methanol:NH_4OH (65:35:5, v/v). We determined the distribution of radioactivity on the chromatograms from area or zonal scans by liquid scintillation spectrometry. Phosphorus was measured by the procedure of Rouser et al. (23). Hydrolysis of the phospholipid (24) containing IPE was achieved with phospholipase C from Bacillus cereus (Grand Island Biological Co.) and diacylglycerols were extracted from the hydrolysate with hexane:diethyl ether (1:1, v/v). Phospho-IPE was hydrolyzed by adding concentrated HCl to the aqueous phase to give a concentration of 6 N; the tube was sealed and heated 16 hr at 105°C. Water was removed and the IPE·HCl converted to the free base by addition of ethanol saturated with KOH, using a trace of phenolphthalein as an indicator. The free base was analyzed by TLC in two solvent systems – chloroform:methanol:acetic acid:H_2O (50:25:8:4, v/v) and chloroform:methanol:NH_4OH (65:35:10, v/v), as well as by gas-liquid chromatography using a 3'x1/8" column packed with 3% XE-60 on 80-100-mesh Gas Chrom Q. The column was temperature programmed from 50-100°C at 5°C/min with a helium flow of 20 ml/min. An effluent splitter in combination with a cold trap at 0°C was used to collect the labeled component corresponding to the retention of IPE. Procedures for the formation of isopropylidene derivatives of the alkylglycerols (25) and their subsequent analysis by gas-liquid chromatography (26) have been described.

RESULTS AND DISCUSSION

L-M cell monolayers incubated with 10 mM IPE for 24 hr contained about 30% fewer cells than the controls. The lower cell number may be due to a change in growth rate, since the viability of the cells that had been treated with IPE was >90% as determined by eosin dye exclusion.

[1] TLC: thin-layer chromatography



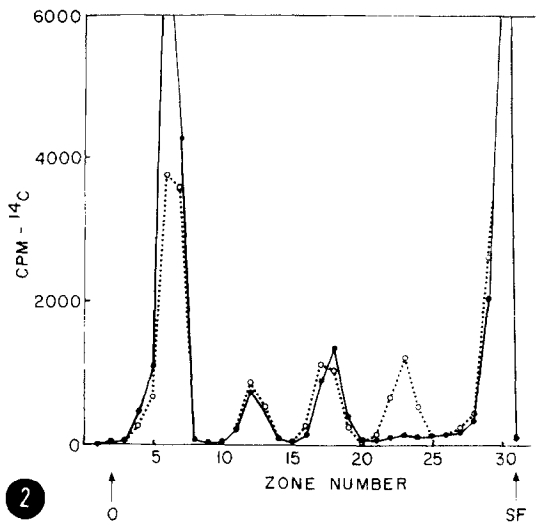


Fig. 1. Accumulation of phospholipid-X in L-M cells maintained for 48 hr in media containing IPE (10 mM).

Fig. 2. Zonal scans of TLC plates developed in chloroform:methanol:acetic acid:H_2O (50:25:8:2, v/v); each zone number corresponds to a 5-mm increment. Aliquots of the total lipids from L-M cells incubated 4 hr with [1-^{14}C]palmitic acid (3.8 μCi) in normal media (●—●) or media containing 10 mM IPE (o---o). Peaks correspond to: phosphatidylcholine (zones 5-8), phosphatidylserine/inositol (zones 10-15), phosphatidylethanolamine (zones 16-19), phospholipid-X (zones 21-25), and neutral lipids (zones 28-31). O and SF indicate the location of the origin and solvent front.

Thin-layer chromatograms of the phospholipids from the L-M cells grown in the presence of IPE for 24 hr showed a new phospholipid (referred to as X) that migrated at an R_f slightly higher than phosphatidylethanolamine in either chloroform:methanol:acetic acid (50:25:8, v/v) or chloroform:methanol:ammonium hydroxide (65:35:5, v/v). Figure 1 shows that the amount of phospholipid-X reached a maximum of about 8% of the lipid phosphorus within 24 hr in the cells grown in media containing the IPE. [1-^{14}C]Palmitic acid was also incorporated into phospholipid-X when the L-M cells were grown in the presence of IPE (Fig. 2). After isolating the labeled phospholipid-X by preparative TLC, it was hydrolyzed with phospholipase C (24). TLC demonstrated all of the radioactivity in the hydrolytic products migrated with 1,2-diacylglycerols.

When monolayers were grown for 24 hr in the presence of ^{14}C-labeled IPE, TLC revealed that ^{14}C was associated with the free base (23%), phosphatidylethanolamine (33%), and phospholipid-X (44%). Incorporation of the ^{14}C from

Table 1. EFFECT OF IPE (10 mM) ON THE INCORPORATION OF VARIOUS LIPID PRECURSORS BY L-M CELLS*

^{14}C-precursor	IPE added simultaneously with ^{14}C-precursor	IPE added 24 hr before ^{14}C-precursor
	% of average control value	
Choline	29 ± 2.6	22; 27
Ethanolamine	56 ± 1.3	22; 22
Palmitic acid	101 ± 12	117 ± 10
Hexadecanol	Not done	102; 98

* All values expressed as the average ± standard deviation of at least four samples, except when given as single numbers the values represent duplicate flasks. The following precursors were incubated for 4 hr: [1,2-^{14}C]choline (3.0 μCi); [1,2-^{14}C]ethanolamine (6.0 μCi); [1-^{14}C]palmitic acid (2.5 μCi); and [1-^{14}C]hexadecanol (3.2 μCi).

Table 2. EFFECT OF IPE ON THE DISTRIBUTION OF INCORPORATED [1-^{14}C]PALMITIC ACID OR [1-^{14}C]HEXADECANOL IN LIPIDS OF L-M CELLS*

	From palmitic acid		From hexadecanol	
Lipid fraction	Control	IPE	Control	IPE
Alkyldiacylglycerols	1.9; 1.8	14; 14	3.2; 2.8	26; 28
Triacylglycerols	14; 14	26; 27	9.0; 8.7	15; 15
Phospholipids	81; 82	52; 52	86; 87	55; 54

* The cells were incubated for 24 hr with IPE (10 mM) and then the labeled palmitic acid (2.5 μCi) or hexadecanol (3.2 μCi) was added and the cultures incubated for another 4 hr. Values are the percent of total ^{14}C distributed in lipid products; radioactivity found in the TLC areas corresponding to the labeled precursors was not included in the total ^{14}C.

IPE into phosphatidylethanolamine presumably occurs by enzymatic removal of the isopropyl moiety. The labeled base group released by hydrolysis from purified phospholipid-X was found to co-chromatograph with IPE by TLC and gas-liquid chromatography. These results are the basis for identifying phospholipid-X as 1,2-diacyl-sn-glycero-3-phosphoisopropylethanolamine.

Whereas IPE inhibited the incorporation of both choline and ethanolamine by L-M cells, no significant effect was seen on the incorporation of either palmitic acid or hexadecanol into the total cellular lipids (Table 1). In

both the control and experimental samples, the radioactivity from [1,2-^{14}C]-choline was found only in sphingomyelin and phosphatidylcholine and the label from [1,2-^{14}C]ethanolamine was found only in the phosphatidylethanolamine.

Little difference was seen in the distribution of radioactivity from [1-^{14}C]palmitic acid among the lipid classes of the cells when the palmitic acid and the unlabeled IPE were added simultaneously to the tissue cultures. However, if the cells were preincubated for 24 hr in media containing IPE and then [1-^{14}C]palmitic acid was added, significant changes occurred in the distribution of radioactivity among different lipid classes when compared to the controls (Table 2). Not only was a greater proportion of radioactivity found in the triacylglycerols of the treated cells, but a substantial quantity of the ^{14}C was associated with the alkyldiacylglycerols, a lipid class produced in only low amounts by the control cultures of L-M cells when grown as monolayers (19). In a separate experiment [1-^{14}C]hexadecanol (precursor of the O-alkyl moieties) was substituted for the [1-^{14}C]palmitic acid; again the shift of radioactivity into the neutral lipids was observed in the presence of IPE and an even greater amount of the ^{14}C from the fatty alcohol was found in the alkyldiacylglycerols (Table 2).

The alkyldiacylglycerols isolated from the cells pretreated with IPE and then incubated with [1-^{14}C]hexadecanol or [1-^{14}C]palmitic acid were chemically reduced with Vitride. When these reduction products were analyzed by TLC, 60% of the labeled hexadecanol and 20% of the labeled palmitic acid was associated with the alkylglycerols. The remaining radioactivity in the products formed by Vitride reduction was found in the fatty alcohols (formerly the acyl groups before reduction with Vitride). Gas-liquid chromatographic analysis revealed that the 16:0-alkyl moiety of the alkyldiacylglycerols, from the IPE-pretreated cells incubated with [1-^{14}C]hexadecanol, contained >90% of the radioactivity.

Total phospholipids, isolated from the cells that had been incubated with [1-^{14}C]hexadecanol, were also reduced with Vitride. Analysis of the Vitride-reduction products by TLC showed that the amount of labeled alkylglycerols in the controls and the cells pretreated with IPE was essentially the same (7.0% vs. 5.7%, respectively). These results indicate that even though the 1-alkyl-2-acyl-sn-glycerols are precursors of both alkyldiacylglycerols (27) and alkyl-type phospholipids (28), selective acylation of 1-alkyl-2-acyl-sn-glycerols to form alkyldiacylglycerols occurs in the IPE-pretreated cells. The increased incorporation of [1-^{14}C]palmitic acid and [1-^{14}C]hexadecanol into alkyldiacylglycerols by L-M cells preincubated with IPE suggests that this amino-alcohol not only inhibits the biosynthesis of phospholipids but also affects the oxidoreductase that forms fatty alcohols from acyl-CoAs (29).

Unnatural amino-alcohol analogs such as IPE that are incorporated into

membrane phospholipids should prove to be useful probes for determining the various functional and structural roles of lipids in membranes. The effect of IPE on the metabolic pathways responsible for the biosynthesis of specific glycerolipid classes suggest that amino-alcohols could be extremely important regulatory molecules in lipid metabolism.

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