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REPLACEMENT OF MOUSE LM FIBROBLAST CHOLINE BY A SULFONIUM ANALOG

EFFECTS ON MEMBRANE PROPERTIES AS DETERMINED BY VIRUS PROBES

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Summary

A sulfonium analog of choline ('sulfocholine', a natural phospholipid constituent of diatoms) was metabolically incorporated into mouse LM fibroblasts cultured in serum-free medium. Subconfluent cultures of LM cells were able to utilize sulfocholine as sole choline source and to increase in cell number for 3 days of incubation; thereafter a decrease in cell number was observed. In contrast, cultures of LM cells seeded to confluency showed no decrease in cell number up to at least 10 days when maintained, with daily medium changes, in medium containing either choline or the sulfonium analog. Such confluent cultures, maintained for 7 days in sulfocholine-containing medium, showed virtually complete replacement of cellular phosphatidylcholine and greater than 50% replacement of cellular sphingomyelin by their respective sulfonium analogs. The functional exchangeability of natural phosphatidylcholine and sphingomyelin with their sulfonium analogs to participate in normal cell membrane-mediated activities was demonstrated by comparatively assaying the abilities of sulfocholine- and choline-maintained cells to incorporate and replicate certain animal viruses known to possess membrane-dependent steps in various phases of their replication cycles. No difference was detected between the abilities of sulfocholine- and choline-maintained cells to take up vesicular stomatitis virus or mengo virus, or to replicate vesicular stomatitis virus, mengo virus or mouse hepatitis virus.

Introduction

Polar head groups of membrane phospholipids are a diverse class of structure varying in size and charge. Accordingly, they possess properties which may contribute to the overall topography of biological membranes as well as imparting distinct influences on membrane 'fluidity' [1]. Despite the fact that specific phospholipid classes may be characteristically associated with a given organism, tissue or subcellular structure, the significance of phospholipid diversity as related to biological function remains unclear. Morever, evolution has provided a rich spectrum of organisms, some divergent in their component phospholipids but, nonetheless, in possession of lipid membranes all fulfilling similar basic functions, e.g., provision of a semipermeable barrier, participation in transport processes and provision of a semifluid matrix for structural support of integral and peripheral membrane proteins. Thus, although in the majority of eukaryotes, the major membrane phospholipid component is phosphatidylcholine (lecithin), several species, notably the phytoflagellate, *Ochromonas danica* [2], and the diatom, *Nitzschia alba* [3], have evolved their own functional analogs of lecithin.

Recent techniques of phospholipid head-group manipulation by cell culture in the presence of choline analogs [4] have begun to provide some insight as to the precise molecular conformations and charge characteristics required of phospholipid polar structures in order that they may participate in a biologically functional membrane. The present report describes the cell-directed incorporation of a sulfonium analog of choline, a major phospholipid constituent of the diatom, *N. alba* [3], into component phospholipids of cultured mouse LM fibroblasts. In order to probe the effects of choline substitution on cell membrane function, we have chosen to study the penetration and replication of selected animal viruses which are known to interact with host cell membranes at various stages in their growth cycles. Similar techniques using viruses as membrane probes have proven to be sensitive indicators of altered membrane characteristics (e.g., Refs. 5 and 6).

Materials and Methods

Radioisotopes, [1,2-^{14}C]choline chloride (spec. act. 10 mCi/mmol) and [^{35}S]mercaptoethanol (spec. act. 8 mCi/mmol) were obtained from New England Nuclear and Amersham, respectively. Sulfocholine was synthesized as previously described [7].

The LM strain of mouse fibroblasts [8] was propagated in the medium described by Higuchi [9]. Choline or sulfocholine supplementation was carried out at concentrations of 40 μg/ml. Cell growth in the presence or absence of choline or sulfocholine was monitored by seeding 100-mm petri dishes each with $5 \cdot 10^5$ cells and incubating at 37°C in 10 ml of medium. At daily intervals, medium was removed and attached cells dissociated with trypsin for determination of cell number by hemocytometer count.

For labeling studies, confluent 35-mm plates of LM cells were incubated for 24 h at 37°C in choline-free medium supplemented with 0.14 μmol/ml of either [^{14}C]choline (10 mCi/mmol) or [^{35}S]sulfocholine (2 mCi/mmol). For

the determination of replacement of lipid choline by exogenous sulfocholine, confluent cultures in 35-mm plates were maintained at 37°C for 24 h in choline-containing medium and then subjected to further incubation at 37°C with daily changes of sulfocholine-containing medium. Cells were harvested by scraping in phosphate-buffered saline [10], pelleted by centrifugation for 10 min at 650 × *g* and extracted with $CHCl_3/CH_3OH$ according to the method of Folch et al. [11]. The filtered extracts were dried under a stream of N_2 and applied to Sil G25 (Brinkmann) thin-layer plates for chromatographic development in $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v). Lipids were detected either by autoradiography or by charring with H_2SO_4 and heat.

Prior to virus-infection experiments, monolayer cultures of LM cells, grown to confluency in 60-mm plates, were maintained for a further 8 days in medium containing either choline or sulfocholine. For the determination of virus penetration, monolayers were inoculated each with 200 plaque forming units of vesicular stomatitis virus or mengo virus. After adsorption for 1 h at 37°C, unadsorbed inoculum was removed and the cells overlaid with 6 ml of Eagle's minimal essential medium [12] containing 10% fetal calf serum and 0.5% methyl cellulose (4000 cps). Incubation proceeded at 37°C for 24 h. Monolayers were subsequently fixed with HCHO and stained with 0.1% crystal violet for visualization of plaques.

For assays of virus replication, confluent 60-mm plate cultures of LM cells, pretreated for 8 days as above with either choline or sulfocholine medium, were inoculated at a multiplicity of unity with vesicular stomatitis virus, mengo virus or mouse hepatitis virus, type JHM [13]. After 1 h adsorption at 37°C, unadsorbed inoculum was removed and the monolayers washed three times with phosphate-buffered saline. The cultures were then overlaid with 6 ml of the medium as described by Higuchi [9] containing either choline or sulfocholine and incubated for 24 h at 37°C. Aliquots were removed from the supernatant media and assayed for virus by plaque assay on the L-2 strain of mouse fibroblasts [14].

Results

Growth of LM cells in choline- or sulfocholine-containing medium

In order to ascertain comparatively the ability of actively growing LM cells to utilize sulfocholine or choline as essential medium component, cells were seeded at $5 \cdot 10^5$ cells/100-mm petri dish. At this cell density, growth in the serum-free medium described by Higuchi [9], supplemented with choline, was initially slow but, within 4 days incubation at 37°C, resulted in a confluent monolayer containing approx. $5 \cdot 10^6$ cells/dish (Fig. 1). Comparable rates of growth have been noted by others [4].

Cells incubated in medium containing sulfocholine showed growth similar to that of choline-grown cells for the first 2 days of culture (Fig. 1); on the third day, cells maintained in sulfocholine showed a decreased growth rate as compared to those maintained in normal choline medium; by day four, cultures grown in sulfocholine showed a decline in cell number. These observations are perhaps analogous to results from animal experiments conducted by Maw and Du Vignaud [15] who demonstrated the lipotropic nature of sulfocholine but

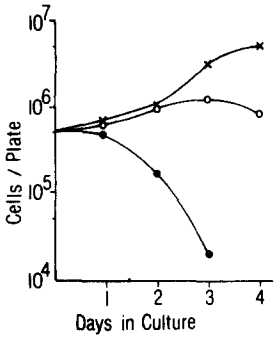

Fig. 1. The effect of culture in choline-free (●———●), choline (40 μg/ml) (X———X), or sulfocholine (40 μg/ml) (○———○) media on cell number of LM cell cultures. Replicate 100-mm plates seeded at $5 \cdot 10^5$ cells/plate were maintained in the above media and the number of attached cells determined after 1, 2, 3 or 4 days by hemocytometer count of trypsinized cultures.

noted that it could not replace choline as a dietary requirement for rats.

For the subsequent experiments described in this report, LM cells were seeded at a density of $5 \cdot 10^6$ cells/100-mm petri dish (or an equivalent cell number for 35- or 60-mm dishes). At this density, cultures were confluent in a matter of hours and, with daily medium changes, showed no decrease in cell number for up to 10 days culture in either choline or sulfocholine medium.

Incorporation of [^{35}S]sulfocholine into LM cell phospholipids

Thin-layer chromatography of lipid extracts prepared from monolayer cultures of LM cells grown for 24 h in the presence of either [^{14}C]choline or [^{35}S]-sulfocholine revealed, in both cases, two major phospholipid species (Fig. 2). By cochromatography with authentic lipid standards, the [^{14}C]choline-labeled phospholipids were identified as phosphatidylcholine and sphingomyelin. Because of the presence of two major subpopulations of fatty acid substituents, sphingomyelin is generally observed as a double spot on thin layer chromatography [16].

The major phospholipid labeled with [^{35}]sulfocholine was identified as phosphatidylsulfocholine by cochromatography with authentic phosphatidyl-sulfocholine isolated from the diatom, *N. alba* [3]. As shown previously [3], the sulfocholine analog of phosphatidylcholine has a slightly higher mobility on thin-layer chromatography than does normal lecithin. The remaining ^{35}S-labeled phospholipid detected in the LM cell lipid extract was inferred to be the sulfonium analog of sphingomyelin (double spot).

Replacement of cellular phosphatidylcholine and sphingomyelin by their sulfonium analogs

In order to determine the extent of replacement of cellular lecithin and sphingomyelin by sulfocholine-containing analogs, monolayer cultures of LM cells were maintained for 24 h in choline medium and then subsequently in sulfocholine medium which was changed daily. Cultures were harvested periodically and processed for lipid extraction. Chromatography of the lipid extracts (Fig. 3) showed that significant replacement of cellular phosphatidylcholine by

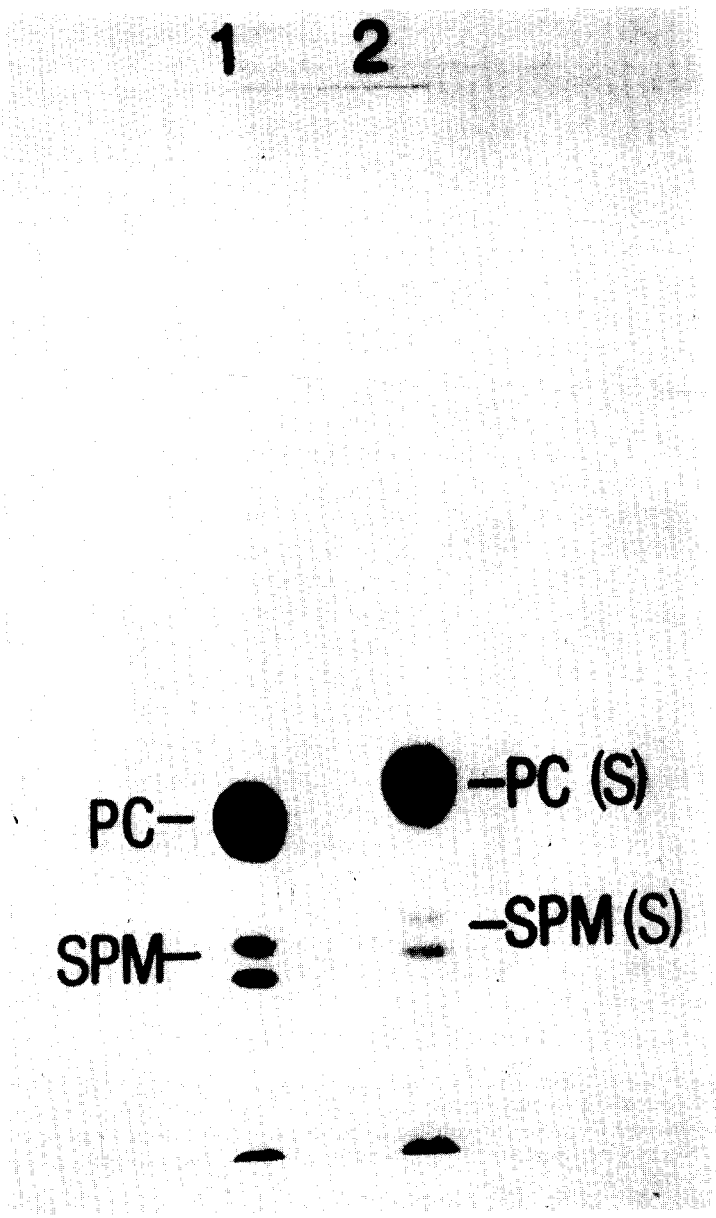

Fig. 2. Autoradiogram of chromatographed [^{14}C]choline- (lane 1) or [^{35}S]sulfocholine- (lane 2) labeled lipids of LM cells. Cells were labeled for 24 h as described in Materials and Methods and the lipid extracts resolved by silica gel thin-layer chromatography in $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v). PC, phosphatidylcholine; PC(S), sulfonium analog of phosphatidylcholine; SPM, sphingomyelin; SPM(S), sulfonium analog of sphingomyelin.

its sulfonium derivative had occurred by 3 days incubation in sulfocholine-containing medium. Complete replacement of phosphatidylcholine, however, was not achieved until about 7 days of culture in the presence of sulfocholine. Replacement of cellular sphingomyelin occurred at a slower rate but was at least 50% complete after 7 days.

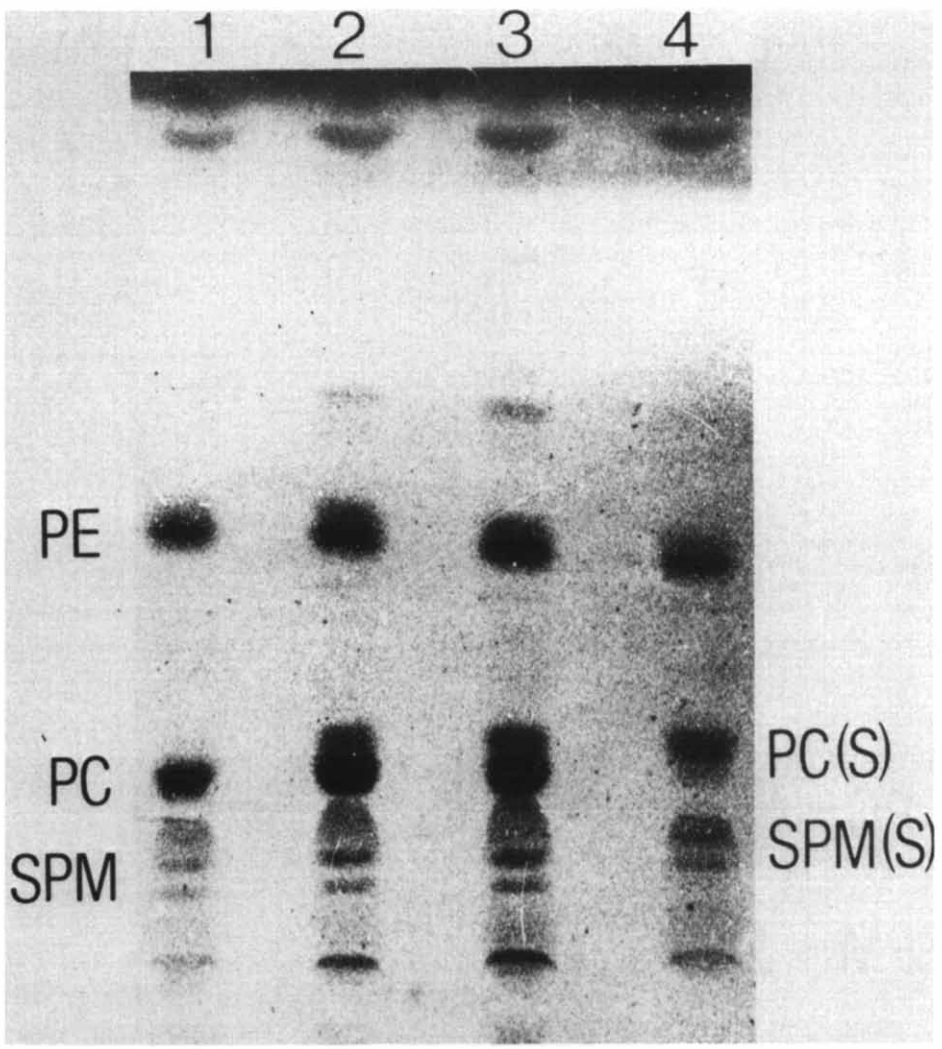

Fig. 3. Thin-layer chromatogram of LM cell lipid extracts developed on silica gel in $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v). Lane 1, cells maintained in choline medium; lane 2, cells maintained for 3 days in sulfocholine medium; lane 3, cells maintained for 5 days in sulfocholine medium; lane 4, cells maintained for 7 days in sulfocholine medium. Lipids were visualized by H_2SO_4 spray and heat-charring. Abbreviations as in Fig. 2; PE, phosphatidylethanolamine.

Penetration and replication of animal viruses in LM cells grown in choline or sulfocholine medium

For all virus experiments, confluent cultures of LM cells were maintained for 8 days in medium supplemented with either choline or sulfocholine and were subjected to daily medium changes. Under these conditions (Fig. 3, lane 4) the vast majority of cellular phosphatidylcholine and at least one-half of the sphingomyelin may be replaced by their sulfonium analogs.

In order to test the ability of cells, in which the phospholipid composition had been modified by sulfocholine supplementation, to participate in the up-

TABLE I

UPTAKE OF VESICULAR STOMATITIS VIRUS AND MENGO VIRUS BY LM CELLS MAINTAINED IN MEDIUM CONTAINING EITHER CHOLINE OR SULFOCHOLINE

Confluent monolayers of LM cells in 60-mm petri dishes maintained for 8 days in either sulfocholine or choline medium were inoculated each with 200 plaque forming units of either vesicular stomatitis virus or mengo virus. Cultures were incubated under a methylcellulose-containing overlay medium for 24 h at 37°C and subsequently stained for plaques. The results are expressed as the mean ± S.D. from determinations from three replicate dishes.

Virus inoculum	Number of plaques	
	Choline-cultured cells	Sulfocholine-cultured cells
Vesicular stomatitis virus	190 ± 16	203 ± 19
Mengo virus	181 ± 23	169 ± 24

take of animal viruses, confluent cultures of LM cells pretreated for 8 days with either choline or sulfocholine medium were challenged with an inoculum of 200 plaque forming units of either vesicular stomatitis virus or mengo virus. The production of visible plaques after an incubation period of 24 h was then taken as evidence for foci of cell lysis and, therefore, of effective virus penetration. Results, shown in Table I, show that in neither the case of vesicular stomatitis virus nor mengo virus infection was plaque production significantly altered by prolonged pretreatment of the cell cultures with sulfocholine. Plaque size was also visually indistinguishable between infected choline- and sulfocholine-cultured cells.

The ability of certain viruses to replicate in LM cells in which phospholipids

TABLE II

REPLICATION OF VESICULAR STOMATITIS VIRUS, MENGO VIRUS AND MOUSE HEPATITIS VIRUS IN LM CELLS MAINTAINED IN MEDIUM CONTAINING EITHER CHOLINE OR SULFOCHOLINE

Confluent monolayers of LM cells in 60-mm petri dishes maintained for 8 days in either choline or sulfocholine medium were inoculated at a multiplicity of infection of unity with vesicular stomatitis virus, mengo virus or the JHM strain of mouse hepatitis virus. After adsorption, excess inoculum was removed and the cultures incubated in appropriate medium for 24 h at 37°C. Aliquots were taken from the supernatant medium and quantitated for virus by plaque assay.

Virus inoculum	Virus titer (plaque forming units/ml)	
	Choline-cultured cells	Sulfocholine-cultured cells
Vesicular stomatitis virus		
Expt. 1	$1.1 \cdot 10^7$	$2.1 \cdot 10^7$
Expt. 2	$1.9 \cdot 10^7$	$2.6 \cdot 10^7$
Expt. 3	$0.9 \cdot 10^7$	$1.0 \cdot 10^7$
Expt. 4	$1.1 \cdot 10^7$	$0.7 \cdot 10^7$
Mengo virus		
Expt. 1	$6.5 \cdot 10^7$	$4.8 \cdot 10^7$
Expt. 2	$9.0 \cdot 10^7$	$8.8 \cdot 10^7$
Expt. 3	$7.2 \cdot 10^7$	$7.5 \cdot 10^7$
Mouse hepatitis virus		
Expt. 1	$2.6 \cdot 10^3$	$2.6 \cdot 10^3$
Expt. 2	$1.3 \cdot 10^3$	$2.0 \cdot 10^3$
Expt. 3	$0.8 \cdot 10^3$	$0.7 \cdot 10^3$

had been modified by culture in sulfocholine was tested by inoculating confluent cultures with vesicular stomatitis virus, mengo virus or the JHM strain of mouse hepatitis virus. After extensive removal of inoculum by repeated washing, virus replication, as assayed after 24 h by plaque assay of the supernatant medium, was found to be essentially the same in sulfocholine-grown cells as those maintained in choline medium (Table II).

It may be noted, incidentally, that virus titers produced by JHM-infected LM cells are considerably lower than those observed in the related L-2 line of mouse fibroblasts [17]. The difference in JHM titer between infected LM and L-2 cells is not merely a consequence of culture in serum-free medium, since even in the presence of 10% fetal calf serum, JHM-infected LM cell cultures produce approximately 100-fold less virus progeny than correspondingly infected L-2 cultures (unpublished observations).

Discussion

The sulfonium analog of choline, being very similar in size and charge, is taken up and incorporated into cellular phospholipid as is natural choline. Virtual replacement of cell phosphatidylcholine by its sulfonium analog can be achieved after 7 days of culture in sulfocholine-containing medium; displacement of cell sphingomyelin occurs at a slower rate, most likely reflecting a slower turnover rate for this phospholipid as compared to phosphatidylcholine [18]. Similarly, Sim and Pasternak [19] reported a much lower rate of incorporation of a phosphonium analog of choline into sphingomyelin than into lecithin of cultured cells.

The use of viruses as probes for various aspects of membrane function is advantageous, since may of the processes associated with various events in virus replication and membrane interaction have been characterized physically and/or biochemically. Virus binding to a susceptible cell requires a cell surface receptor [20], the accessibility and motional freedom of which would be expected to be subject to similar fluidity-dependent constraints which affect other membrane-bound receptor molecules [21]. Attachment of adenovirus, for example, has been reported to show a change in activation energy at the transition temperature of the cell membrane lipid [22].

Uptake of viruses such as vesicular stomatitis virus [23] or mouse hepatitis virus [24] may be taken as a measure of the ability of the cell plasma membrane to perform pinocytosis (or viropexis). The pinocytic process itself is an endothermic event and has been shown to be dependent on the lipid environment present in the invaginating cell membrane [5]. In the case of the picornavirus, mengo, at least partial uncoating of the virion may occur at the cell surface [25] wherupon infectious virion RNA and/or viral substructures penetrate the plasma membrane by an as yet unclear mechanism. The results of the present study suggest that sulfocholine analogs of phosphatidylcholine and sphingomyelin are functionally interchangeable with their natural counterparts in mediating the membrane processes involved in viral pinocytosis and uptake of infectious viral genome.

The replication strategies of the three viruses used to probe the effects of sulfocholine/choline substitution in LM cells may be highlighted very briefly.

Both vesicular stomatitis virus [26] and mouse hepatitis virus [24] assemble by budding mechanisms, involving regional invagination of host plasma membrane and endoplasmic reticulum, respectively, around progeny nucleocapsids. Both viruses contain glycoprotein 'spikes' which, prior to the assembly event, must be inserted into host lipid membrane. In contrast, mengo virus, a non-enveloped virion, does not mature by a budding mechanism but, nevertheless, appears to utilize cellular endoplasmic reticulum as an assembly site for formation of viral substructures [27]. The ability of sulfocholine-cultured LM cells to replicate efficiently these three viruses may be taken as evidence for the biologically competent participation of sulfonium phospholipids in the complex membrane interactions with various viral polypeptides and structural intermediates of viral assembly. For comparison, it is interesting to note that Maeda et al. [6] recently documented small but discernible differences in the replication of vesicular stomatitis virus when the host LM cell was cultured in the presence of the choline analogs, ethanolamine, *N*-methylethanolamine and *N,N'*-dimethylethanolamine.

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