The Metabolism of the Phosphonium Analogue of Choline in Cultured Cells

A USEFUL NUCLEAR-MAGNETIC-RESONANCE PROBE FOR MEMBRANE PHOSPHATIDYLCHOLINE

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1. Replacement of choline by the phosphonium analogue does not affect the growth rate of P815Y, NIL, 3T3 and SV40/3T3 cells in culture. 2. The fatty acid composition of the resulting phosphonium phosphatidylcholine is similar to that of phosphatidylcholine. 3. The rate of synthesis and degradation of phosphatidylcholine and of the phosphonium analogue are similar. 4. Phospholipid-exchange protein does not distinguish between phosphatidylcholine and the phosphonium analogue. 5. By contrast, incorporation of phosphonium choline into sphingomyelin occurs to only a minor extent. 6. It is concluded that, since the enzymes involved in the turnover of phosphatidylcholine do not discriminate between quaternary N and quaternary P in the polar head-group region, phosphonium choline should prove to be a useful probe for ^{31}P nuclear-magnetic-resonance (n.m.r.) studies of natural membranes.

Phosphonium choline, in which the nitrogen atom of choline is replaced by a phosphorus atom (Fig. 1), gives a unique ^{31}P n.m.r. (nuclear-magnetic-resonance) signal distinct from that of phosphate (Edwards, 1973). The analogue appears as phosphonium phosphatidylcholine in rats (Edwards, 1973) and in *Neurospora crassa* (J. Grayheb, unpublished work) that have been fed on phosphonium choline, without any apparent signs of toxicity. It is therefore a potentially useful probe of membrane structure, in that it is readily detectable, yet appears to cause minimal perturbation of cell membranes.

In the present paper we give details of its metabolism by a variety of normal and of transformed cells in culture. A brief report of this work has already appeared (Pasternak *et al.*, 1975).

Experimental

Materials

Phosphonium choline chloride was synthesized by the method of Renshaw & Bishop (1938) as described by Edwards (1973). Phosphonium [^{14}C]-choline chloride was synthesized as follows (Edwards, 1973). 2-Bromo[1,2- ^{14}C]ethanol (The Radiochemical Centre, Amersham, Bucks., U.K.) (1 mCi; 0.1 mmol), with 2-chloroethanol (3.9 mmol) as carrier, was treated with excess of trimethylphosphine (0.6 ml) and dry ethanol (0.5 ml) in a sealed glass bomb at 100°C for 6 h in an atmosphere of N_2 . The mixture was cooled, and ethanol, excess of trimethylphosphine and any unchanged 2-chloroethanol were removed by heating (100°C) under decreased pressure [1.3 kPa (10 mmHg)]. The resulting white solid was dissolved in water and passed down a column of Amberlite IRA-400 (Cl⁻ form). The effluent and the water washes were dried under decreased pressure. More than 95% of the radioactivity of the resulting material migrated with phosphonium choline during paper electrophoresis in 1% (w/v) ammonium carbonate, pH 8.9. Phosphonium choline chloride used as standard was synthesized and donated by Dr. R. G. Edwards (Scheie Eye Institute, Philadelphia, Pa., U.S.A.).

Fig. 1. Structural formulae of (a) choline and (b) the phosphonium analogue of choline

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Cell culture

3T3- and SV40-transformed 3T3 cells (Balb/c) and NIL 2 foetal hamster cells were grown on plastic dishes in Dulbecco's modified minimum essential medium (Dulbecco & Freeman, 1959; 0.03M-choline) with choline replaced by an equivalent concentration of the phosphonium analogue where indicated (phosphonium Dulbecco's medium). 3T3 cells were grown in the presence of 15% (v/v) foetal calf serum and other cell lines with 10% (v/v) calf serum.

P815Y mouse mastocytoma cells were grown in suspension culture in Fischer's medium (Fischer & Sartorelli, 1964) with 8% (v/v) horse serum. All cultures contained streptomycin sulphate (5mg/100ml) and benzylpenicillin (30mg/100ml) (Glaxo Laboratories, Greenford, Middx., U.K.). Cell culture media and sera were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., from Gibco-Biocult, Paisley, Scotland, U.K., or from Tissue Culture Services, Slough, Bucks., U.K. All cells were grown at 37°C.

Incorporation and turnover

Monolayer cells grown in Dulbecco's medium or phosphonium Dulbecco's medium for six to eight generations were passaged in Dulbecco's medium containing phosphonium [^{14}C]choline chloride (0.04–0.1 $\mu\text{Ci/ml}$; final specific radioactivity 170 $\mu\text{Ci/mmol}$) and [$\text{Me-}^3\text{H}$]choline chloride (0.15–1.0 Ci/ml ; final specific radioactivity 5–35 mCi/mmol) and were grown for 3 days. The cells were then washed and passaged in Dulbecco's medium or phosphonium Dulbecco's medium and again grown to confluency. Samples of medium and cells (harvested by scraping with a Teflon 'policeman') were taken at intervals.

P815Y cells were grown in suspension culture for 3 days in Fischer's medium containing phosphonium [^{14}C]choline chloride and [$\text{Me-}^3\text{H}$]choline chloride as described for monolayer cultures. Alternatively, suspension cultures were pulsed with either phosphonium [^{14}C]choline chloride (0.04 $\mu\text{Ci/ml}$; final specific radioactivity 170 Ci/mmol) and [^{32}P]orthophosphate (0.2 $\mu\text{Ci/ml}$) or [$\text{Me-}^3\text{H}$]choline chloride (0.04 $\mu\text{Ci/ml}$; final specific radioactivity 150 $\mu\text{Ci/mmol}$) and [^{32}P]orthophosphate (0.2 $\mu\text{Ci/ml}$). After 16h the cells were harvested by centrifuging, and then suspended in Fischer's medium and grown for a further 17h. Appropriate dilutions were made to maintain exponential growth at all times.

All cells were extracted by the method of Folch *et al.* (1957) and the lipid layer was either assayed directly or was separated by two-dimensional t.l.c. on silica gel H (Merck, Darmstadt, Germany) as follows: chloroform/methanol/7 M- NH_3 (46:18:3, by vol.) in the first dimension, followed by chloroform/methanol/acetic acid/water (50:50:1:4, by vol.) in

the second dimension. This method separates phosphatidylcholine from the phosphonium analogue (Sim *et al.*, 1975). Phospholipids were detected by exposure to I_2 vapour, by spraying with Rhodamine B/2,7-Dichlorofluorescein (Jones *et al.*, 1966) and observing under u.v. light, or by radioautography. Appropriate areas were removed, eluted with ethanol/chloroform/water (10:3:2, by vol.) and were assayed directly or were rechromatographed in chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) (Skipski *et al.*, 1964).

Fatty acid composition

Phosphatidylcholine and phosphonium phosphatidylcholine, separated by two-dimensional chromatography, were evaporated to dryness under N_2 at 37°C and heated with benzene (0.4ml), methanol (1.6ml) and approx. 10M- H_2SO_4 (0.5 μl) under N_2 in a sealed tube at 70°C for 4h. The methyl esters were extracted with light petroleum (b.p. 40–60°C), dried under N_2 at room temperature (20°C) and re-suspended in acetone. Samples containing approx. 0.5 μg of phospholipid P were applied to a column of 15% (w/w) polyethylene glycol succinate (80–100 mesh). The column temperature in the Pye Unicam gas-liquid chromatogram was 173–174°C and the injection temperature was 250°C. The carrier gas, argon, had a flow rate of 60ml/min. Sub-peak areas were determined graphically or by weighing the tracings of the peaks. Molar response factors were determined by methanolysis of 0.5mmol of standard fatty acids.

Other methods

Phospholipid P was determined by the method of Bartlett (1959) after digestion at 110°C for 24h in constant-boiling HCl. This method of hydrolysis eliminates any contribution of the phosphonium moiety to the phosphorus content. Cellular protein (in the precipitate obtained during the lipid extraction) was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (Sigma, Kingston-upon-Thames, Surrey, U.K.) as standard. Radioactivity was assayed in 1,4-dioxan containing 5g of diphenyloxazole/1 litre and 20g of naphthalene/1 litre with an LKB-Wallac liquid-scintillation counter. Lipid samples were evaporated to dryness before assay. Appropriate corrections for the presence of ^{14}C in the ^3H channel and for efficiency were made.

Results and Discussion

Effect of phosphonium choline on growth

Phosphonium choline is without adverse effect on the growth of cultured cells. Fig. 2 shows the results

with SV40/3T3 cells, which continue to grow normally for more than eight generations in medium containing phosphonium choline as choline source. Similar results have been obtained with 3T3, NIL and P815Y cells. This may be contrasted with the inhibition in growth rate of LM cells, observed 4-5 days

Fig. 2. Effect of phosphonium choline on cell growth SV40/3T3 cells were grown in Dulbecco's medium (○) or phosphonium Dulbecco's medium (●) supplemented with 10% (v/v) calf serum. Duplicate plates were harvested at the times indicated and the cellular protein concentration was determined as described in the Experimental section.

Table 1. Comparison between fatty acid compositions of phosphatidylcholine and phosphonium phosphatidylcholine

Cells were grown in Dulbecco's medium (N growth medium) or phosphonium Dulbecco's medium (P growth medium) for six to eight generations; each medium contained 10% (v/v) calf serum. Phosphatidylcholine (N) and phosphonium phosphatidylcholine (P) were separated and analysed as described in the Experimental section. The results for SV40 3T3 cells are shown as individual values of two experiments; for NIL cells they are the means ± s.e.m. of three experiments. Tr., Component present in trace amounts (less than 1% of total fatty acids).

Cell line	Growth medium	Phosphatidylcholine	Fatty acid composition (mol%)										Unsat.(%)	
			C _{14:0}	C _{16:0}	C _{16:1}	C _{18:0}	C _{18:1}	C _{18:2}	C _{18:3}	C _{20:0}	C _{20:unsat.}	Others	sat.(%)	
SV40/3T3	N	Total	Tr.	22.9	3.8	16.6	38.7	7.0	1.1	2.0	7.8	Tr.	1.4	
			3.6	24.6	4.7	15.9	34.0	4.3	Tr.	2.7	10.3	Tr.	1.1	
	P	N	2.1	23.0	6.9	17.7	36.6	4.7	Tr.	2.7	6.2	Tr.	1.2	
			3.7	22.3	6.8	13.0	40.1	8.8	2.1	1.5	1.5	Tr.	1.5	
	P	P	Tr.	21.5	5.7	23.0	32.6	1.5	7.5	3.8	4.2	Tr.	1.0	
			2.9	25.0	5.0	19.3	37.1	4.6	3.0	0.9	2.1	Tr.	1.1	
NIL	N	Total	Tr.	31.8	5.0	10.8	39.0	4.9	Tr.	Tr.	4.3	1.6	1.3	
				±1.9	±0.3	±0.9	±1.0	±1.1			±1.4	±0.2		
	P	N	Tr.	27.8	6.5	16.9	27.6	5.6	1.2	Tr.	11.4	1.4	1.2	
				±1.5	±1.1	±2.9	±0.2	±0.7	±0.3		±2.3	±0.1		
	P	P	Tr.	32.3	6.6	13.5	29.3	5.9	Tr.	Tr.	7.0	2.6	1.2	
				±4.5	±0.6	±0.4	±4.0	±1.1			±1.6	±0.4		

after transfer to medium containing monomethyl- or dimethyl-ethanolamine as choline source (Glaser *et al.*, 1974). Cells grown on phosphonium choline as choline source have up to 60% of their phosphatidylcholine replaced by the phosphonium analogue (Pasternak *et al.*, 1975). Failure to replace phosphatidylcholine entirely presumably reflects the presence of choline and phosphatidylcholine in the serum, as well as the extent to which the methylation of ethanolamine (Åkesson *et al.*, 1970) might contribute to the synthesis of phosphatidylcholine.

Nature of phosphatidylcholine sub-species

Growth in the presence of phosphonium choline results in the formation of phosphonium phosphatidylcholine with an essentially similar distribution of fatty acids to that obtained with phosphatidylcholine (Table 1). In general, the phospholipid composition reflects the acyl chain composition of the serum in which cells are grown (R. M. Abra, unpublished work), which confirms previous observations (Bailey *et al.*, 1972). Whatever apparent differences are found (e.g. in C_{18:0}, C_{18:2} and C_{18:3} acids in SV40/3T3 cells) are not general ones (compare C_{18:0}, C_{18:2} and C_{18:3} acids in NIL cells) and are therefore unlikely to be of significance with respect to differences in the head-group region. Insofar as the acyl-chain composition of phospholipids is determined by the necessity to maintain membranes above their phase-transition temperature (Fox, 1972), the results given in Table 1 are entirely consistent with the observation that the phase-transition temperature of distearoyl phosphatidylcholine and its phosphonium analogue are the same (Sim *et al.*, 1975).

Table 2. Isotopic ratios in cells exposed to [^3H]choline and phosphonium [^{14}C]choline

Cells were grown for 3 days in medium with serum supplement containing [^3H]choline (0.15–1.0 $\mu\text{Ci/ml}$; final specific radioactivity 5–35 mCi/mmol) and phosphonium [^{14}C]choline (0.04–0.1 Ci/ml ; final specific radioactivity 170 $\mu\text{Ci/mmol}$), were harvested and extracted, and the phospholipids separated as described in the Experimental section. More than 98% of the ^3H and ^{14}C in the lipid extract was recovered as phosphatidylcholine+sphingomyelin. Ratios are expressed as the mean $\pm$ S.E.M.

Cell line	No. of determinations	$^3\text{H}/^{14}\text{C}$ ratio				
		Pulse medium	Aqueous extract	Total lipid	Phosphatidylcholine	Sphingomyelin
NIL	4	4.0	1.9 ± 0.2	5.7 ± 0.5	4.3 ± 0.4	25.6 ± 2.1
SV40/3T3	5	3.2	2.0 ± 0.2	7.2 ± 0.3	5.4 ± 0.2	32.4 ± 1.3
3T3	3	6.5	4.3 ± 0.7	7.2 ± 0.2	5.0 ± 0.2	31.4 ± 4.6
P815Y	1	11.9	7.5	16.9	17.8	150.0

With phosphatidylcholine analogues lacking one or two *N*-methyl groups, the phase-transition temperature is altered (Vaughan & Keough, 1974), and, as might be expected, so is the fatty acid composition of the phosphatidylcholine analogues (Ferguson *et al.*, 1975).

Turnover of phospholipids

Water-soluble intermediates. Phosphonium choline is taken up and metabolized as effectively as is choline by all the cell lines studied. In fact the isotopic ratio, indicative of the choline/phosphonium choline ratio, in the water-soluble fraction of several cell lines, is somewhat lower than that in the growth medium (Table 2). Since the measurements were made at the end of a 3-day pulse, deviations from the ratio in the medium may reflect differences in the fate of the water-soluble components subsequent to turnover of phospholipids. Certainly the proteins catalysing transport and metabolism up to CDP-choline do not appear to discriminate against the phosphonium analogue. This confirms preliminary results with crude enzyme preparations from rat liver (Edwards, 1973).

Phosphatidylcholine. The extent of incorporation into cellular phospholipids is somewhat less with phosphonium choline than with choline (as revealed by the isotopic ratio; Table 2). This is largely due to an impaired incorporation into sphingomyelin (see below). The total incorporation into phosphatidylcholine is unimpaired and is the same for normal cells (3T3) and for the transformed variant (SV40/3T3) (Table 2). Once incorporated, the phosphonium analogue turns over at the same rate as phosphatidylcholine (Fig. 3), with the biphasic characteristics noted previously (Pasternak & Bergeron, 1970; Pasternak, 1972). The half-life of the unstable component (which is predominantly phosphatidylcholine as opposed to sphingomyelin; Pasternak & Bergeron, 1970) is approximately the same irrespective of whether the cells are pre-grown on choline or on phosphonium choline, and whether they are subsequently transferred to choline- or phosphonium

Fig. 3. Turnover of phospholipids in cells pre-labelled with [^3H]choline and phosphonium [^{14}C]choline

NIL cells were preincubated for 3 days in the presence of [^3H]choline and phosphonium [^{14}C]choline, washed and passaged in Dulbecco's medium with 10% (v/v) calf serum as supplement as described in the text. Duplicate plates were harvested at the times indicated and the specific radioactivities (d.p.m./mg of cell protein) of ^3H (●) and ^{14}C (○) in the lipid extract were determined. Cells continued to grow exponentially throughout the experimental period.

choline-containing medium (Table 3). It should be noted that base exchange (Treble *et al.*, 1970) is unlikely to contribute significantly to the turnover of phosphatidylcholine at the phosphonium analogue, since [^{32}P]phosphate is incorporated and degraded in concert with choline or phosphonium choline (Fig. 4a).

Sphingomyelin. Table 2 shows that all cultures examined discriminate against phosphonium choline

Table 3. *Turnover of phospholipids in cells pre-labelled with [³H]choline and phosphonium [¹⁴C]choline*

Cells were grown in Dulbecco's medium (N grown) or phosphonium Dulbecco's medium (P grown) for six to eight generations. Cells were then exposed to [³H]choline (0.15 μ Ci/ml; final specific radioactivity 5 mCi/mmol) and phosphonium [¹⁴C]choline (0.04 μ Ci/ml; final specific radioactivity 170 μ Ci/mmol) for 3 days, washed and passaged in Dulbecco's medium (N) or phosphonium Dulbecco's medium (P). Samples were removed at intervals over 3 days, during which time exponential growth was maintained. The specific radioactivities of ³H and ¹⁴C in the lipid extract were determined and plotted semi-logarithmically as in Fig. 3; the half-life of the unstable component (initial $t_{\frac{1}{2}}$) was determined. All cultures contained 10% (v/v) calf serum. Values obtained from individual experiments are shown.

Cell line	Resuspending medium	Initial $t_{\frac{1}{2}}$ (h)	
		³ H	¹⁴ C
NIL (N grown)	N	9.2	10.2
	P	8.9; 9.5	7.5; 10.5
SV40/3T3 (N grown)	N	10.7; 10.5	10.0; 11.0
	P	6.9	7.7
SV40/3T3 (P grown)	N	7.4	8.5
	P	8.9	11.0

as far as sphingomyelin synthesis is concerned. Nevertheless a limited incorporation of phosphonium [¹⁴C]choline into sphingomyelin is probably real, since the sphingomyelin area obtained after two-dimensional t.l.c. is partially resolved into choline- and phosphonium choline-containing species. The relative positions of the two components are the same as those of phosphatidylcholine and phosphonium phosphatidylcholine (Sim *et al.*, 1975). Incorporation of phosphonium choline into sphingomyelin may represent the minor of the two postulated pathways for the biosynthesis of sphingomyelin: (1) condensation of CDP-choline with ceramide (Sribney & Kennedy, 1958) and (2) transfer of the phosphorylcholine moiety from phosphatidylcholine directly to ceramide (Marggraf & Anderer, 1974). That the latter pathway operates in the cells under study is indicated by the fact that maximum labelling of sphingomyelin is achieved some hours after that of phosphatidylcholine (see Figs. 4a and 4b). Fig. 4(b) indicates very clearly the relative lack of sphingomyelin synthesis from phosphonium choline. Whether the block is at reaction (1) or (2) requires further investigation.

Phospholipid-exchange proteins. In view of the fact that cultured cells (Pasternak, 1976) contain proteins capable of exchanging phosphatidylcholine between different organelles (Wirtz, 1974), it seemed worth while to test the activity of a partially purified

Fig. 4. *Incorporation of [³H]choline, phosphonium [¹⁴C]choline and [³²P]phosphate into phosphatidylcholine and sphingomyelin*

P815Y cells growing in Fischer's medium with 8% (v/v) horse serum were exposed either to [³H]choline (0.04 μ Ci/ml; final specific radioactivity 150 μ Ci/mmol) and [³²P]phosphate (0.2 μ Ci/ml) or to phosphonium [¹⁴C]choline (0.04 μ Ci/ml; final specific radioactivity 170 μ Ci/mmol) and [³²P]phosphate (0.2 μ Ci/ml) for 16 h. Each culture was resuspended in Fischer's medium containing 8% (v/v) horse serum and allowed to grow for a further 17 h. Samples were taken at intervals and the specific radioactivities (c.p.m./ μ g of phospholipid P) of ³H (○) and the corresponding ³²P (●) or of ¹⁴C (Δ) and the corresponding ³²P (▲) in phosphatidylcholine (a) and in sphingomyelin (b) were determined as described in the Experimental section. The arrow denotes the start of the 'chase' period.

Table 4. Comparison of the activities of phospholipid-exchange protein towards phosphatidylcholine and phosphonium phosphatidylcholine

Expt. 1. Mouse liver microsomal fraction (5.7 μ g of phospholipid P in 0.5 ml of 0.25 M-sucrose/0.1 mM-EDTA, pH 7.3) was incubated with liposomes, prepared from phosphatidylcholine (extracted from Lettuce cells preincubated with [3 H]choline and phosphonium [14 C]choline) as follows. Phosphatidylcholine (165 μ Ci of 3 H/ μ mol of phospholipid P; 2.7 μ Ci of 14 C/ μ mol of phospholipid P) was sonicated, on ice, for 2 min in a conical steel pot with a 250 W, 20 kHz input, in 10 mM-Tris/HCl buffer, pH 7.3, and spun (105 000g for 1 h). A 0.5 ml sample of the clear supernatant (35 μ g of phospholipid P) was used in each incubation. Expt. 2. Rat liver microsomal fraction (28 μ Ci of 3 H/ μ mol of phospholipid P; 3.15 μ Ci of 14 C/ μ mol of phospholipid P) prepared from rat liver slices pre-labelled with [3 H]choline and phosphonium [14 C]choline, was incubated (9.3 μ g of phospholipid P in 0.5 ml of 0.25 M-sucrose/0.1 mM-EDTA, pH 7.3) with 0.5 ml of liposomes (55 μ g of phospholipid P/ml) prepared from unlabelled mouse liver phosphatidylcholine as described for Expt. 1. Phospholipid-exchange protein was partially purified from bovine heart. After incubation of the mixtures (final volume 1.02 ml) for 90 min at 25°C or 37°C, 0.9 ml of ice-cold 0.25 M-sucrose/10 mM-Tris/HCl buffer, pH 7.3, was added and the mixture spun for 1 h at 105 000g. In Expt. 2 the specific radioactivity of the total supernatant was determined. In Expt. 1 the microsomal pellet was washed by re-spinning at 105 000g for 1 h and the specific radioactivity of the resulting pellet measured.

Expt. no.	Temp. (°C)	Phospholipid-exchange protein (μ g/ml)	Labelled component	Final sp. radioactivity of unlabelled component (d.p.m./ μ g of phospholipid P)		3 H/ 14 C ratio
				3 H	14 C	
1	25	—	Liposomes	1200	20	60
	25	20	Liposomes	2840	46	62
	37	—	Liposomes	2210	35	63
	37	20	Liposomes	3300	49	67
2	37	—	Microsomal fraction	400	30	13
	37	10	Microsomal fraction	960	86	11
	37	20	Microsomal fraction	950	94	10

phospholipid-exchange protein against the phosphonium analogue of phosphatidylcholine. Table 4 shows that phosphatidylcholine and phosphonium phosphatidylcholine are exchanged with equal efficiency between sonicated phospholipid vesicles and mammalian liver microsomal particles. Whatever the physiological significance of phospholipid-exchange proteins proves to be, it thus becomes possible to introduce phosphonium choline-containing phospholipids into cell membranes under defined conditions, for subsequent 31 P n.m.r. studies.

Conclusions

The results presented indicate that phosphonium choline is likely to prove at least as useful a probe of membrane structure as 13 C-enriched lipids (Metcalf *et al.*, 1972) or spin-labelled fatty acids (Tourtellotte *et al.*, 1970), in that it is incorporated biosynthetically and causes minimal perturbation. Its advantage lies, not only in the uniqueness of the phosphonium 31 P n.m.r. signal (Edwards, 1973), but also in the fact that it is specifically incorporated into only one phospholipid class, phosphatidylcholine, to any major extent. This is in contrast with the deuterated analogue of choline recently used as an n.m.r. probe by Arvidson *et al.* (1975), which is incorporated into both phosphatidylcholine and sphingomyelin. The discrimination against incorporation of phosphonium choline into sphingomyelin may prove useful in

investigating the biosynthesis of sphingomyelin-enriched membranes such as the plasma membrane.

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