Decreased content of arachidonoyl species of phosphatidylinositol phosphates in pancreas of rats fed on an ethanol-containing diet

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Phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-diphosphate [PtdIns(4,5)P₂] were isolated from the pancreas of rats fed an ethanol-containing liquid diet for 24 days and from the corresponding pair-fed controls. The isolation involved chromatography on a lipophilic anion exchanger in the phosphate form. The species composition was determined by fast-atom bombardment mass spectrometry. The compositions of PtdIns4P and PtdIns(4,5)P₂ were similar to that of PtdIns, with the stearoyl/arachidonoyl species constituting about 32 % of the total, compared with 38 % in PtdIns. PtdIns(4,5)P₂ contained a larger fraction of fully saturated species than PtdIns. The PtdIns species having an arachidonoyl group were about half as abundant in the ethanol-treated as in the control rats. The differences between ethanol-fed and control rats were qualitatively similar for PtdIns, PtdIns4P and PtdIns(4,5)P₂, but were less marked for PtdIns4P and PtdIns(4,5)P₂. The results indicate that the species compositions of PtdIns4P and PtdIns(4,5)P₂ reflect that of PtdIns, and that the changes of PtdIns4P and PtdIns(4,5)P₂ in ethanol-treated rats are secondary to changes in PtdIns.

INTRODUCTION

The composition of phosphatidylinositol (PtdIns) in the pancreas has been found to be markedly changed in rats fed on ethanol-containing diets [1–4]. The content of arachidonoyl-containing species is decreased by about 50 %. Attempts to prevent or mimic this effect by pharmacological [2] or dietary [4] means have not been successful, and the reason for the change and its possible consequences are not known. It might conceivably be related to the mechanism behind alcohol-induced chronic pancreatitis, since PtdIns is a constituent of the cell membranes, acts as an anchor for membrane-bound enzymes [5] and serves as a precursor for the phosphorylated forms of PtdIns, PtdIns4P and PtdIns(4,5)P₂ [6]. Hydrolysis of the latter by the hormone-sensitive phospholipase C gives rise to secondary intracellular events initiated by the released inositol trisphosphate, diacylglycerol and possibly arachidonate [6]. In view of these considerations it was of interest to study whether ethanol feeding affected the species composition of the phosphorylated forms of PtdIns in the pancreas. We describe results obtained in analyses by fast-atom bombardment mass spectrometry (f.a.b.m.s.) of the intact phospholipids, isolated by chromatography on a lipophilic anion exchanger.

MATERIALS AND METHODS

Materials

Ptd[2-³H]Ins4P (6.9 Ci/mmol) and Ptd[2-³H]Ins(4,5)P₂ (4.3 Ci/mmol) were from NEN–Du Pont de Nemours & Co. (Wilmington, DE, U.S.A.). Unlabelled lipid references were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and triethanolamine was from Merck (Darmstadt, Germany). All glassware was siliconized before use.

Lipidex–DEAP (Packard Instrument Co., Downers Grove, IL, U.S.A.) [7] was washed with aq. ethanol and converted to the phosphate form via the hydroxide form by treatment with 0.1 M-H₃PO₄ in 50 % aq. methanol, followed by washing with 50 % aq.

methanol until neutral and finally with chloroform/methanol/water (3:6:1, by vol.). Columns (100 mm × 4 mm) were prepared and used in this solvent.

Animal experiments

Male Sprague–Dawley rats weighing initially 135–160 g were fed on liquid diets for 24 days [8,9]. The alcohol diet, which provides 36 % of the energy from ethanol, and the control diet, with carbohydrate replacing ethanol, were obtained from Bioserv, Inc. (Frenchtown, NJ, U.S.A.). Ethanol was introduced stepwise, reaching its final concentration on day 5. The controls were pair-fed [8], with the ration divided into two equal parts provided in the morning and the afternoon. The control rats increased in weight by 75 ± 20 g, whereas there was no significant weight gain of the ethanol-fed rats. The food was removed on the evening before the rats were killed. The pancreas was removed under ether anaesthesia.

Isolation of phosphoinositides

Each pancreas was immediately homogenized in 3 ml of chloroform/methanol (1:2, v/v), and about 3 MBq of ³H-labelled PtdIns4P or PtdIns(4,5)P₂ was added together with 0.6 ml of 1.2 M aq. HCl prior to extraction with chloroform, as described by Schacht [10]. Methanol was added to the pooled and washed extracts to yield chloroform/methanol (3:2, v/v). This solution was applied to the Lipidex–DEAP column, and most of the lipids were eluted with chloroform/methanol (3:2, v/v) or chloroform/methanol/water (3:6:1, by vol.). Phosphatidylserine and PtdIns were eluted with 0.03 M-H₃PO₄ and unidentified material with 0.1 M-H₃PO₄. PtdIns4P was eluted with 0.25 M-H₃PO₄ and PtdIns(4,5)P₂ with 0.5 M-H₃PO₄ according to eluted radioactivity. All fractions were extracted with 0.4 ml of water/ml of eluate. The upper phase was back-extracted with 0.1 vol. of chloroform. The pooled chloroform phases were washed with 0.4 vol. of 0.5 M-HCl in 50 % aq. methanol.

PtdIns was purified by preparative h.p.t.l.c. using established solvent systems [11].

Abbreviations used: f.a.b., fast atom bombardment; h.p.t.l.c., high performance thin layer chromatography.

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Analytical methods

H.p.t.l.c. Silica gel plates (Merck) were treated with potassium oxalate as described [12], except that 50% aq. ethanol was used as solvent. The plate was washed by developing once with the mobile phase [chloroform/methanol/3.3 M aq. ammonia (43:38:12, by vol.)] [10], and spots were visualized with iodine vapour.

Gas chromatography. The phospholipids were trans-esterified with 200 μ l of 5% BF_3 in methanol for 10 min at 100 °C. After addition of water, the esters were extracted with hexane and analysed by gas chromatography on a 30 m $\times$ 0.32 mm internal diam. fused silica capillary column with a 0.25 μ m layer of DB-23 (J & W Scientific, Folsom, CA, U.S.A.), with helium as carrier gas. The samples were injected on-column, and the temperature was raised from 60 to 160 °C at 25 °C/min and then to 250 °C at 2 °C/min. Dotriacontane was added to samples and blanks and used in corrections for contamination of solvents etc. with fatty acids and for quantifications.

F.a.b.m.s. A VG 7070E double-focusing mass spectrometer equipped with an f.a.b. ion source (used at 6 kV), an Ion Tech atom gun (producing 7 keV Xe atoms) and a VG 11-250 data system (VG Analytical, Manchester, U.K.) was used. The phosphoinositides were dissolved in 20 μ l of chloroform/methanol/water (86:14:1, by vol.) and one-tenth of the sample was applied under a gentle stream of N_2 to the f.a.b. target, already covered with triethanolamine. Spectra of negative ions were recorded between m/z 1200 and m/z 700 at a scan rate of 20 s per decade using multichannel signal-averaging data acquisition. The resolution was 1200 (5% valley) and the total acquisition time for a spectrum was 1–2 min. Calibration and mass measurements were carried out using glycerol. The relative intensities of the quasimolecular ions were used to calculate the species composition, assuming identical molar responses. Corrections were made for contributions due to natural abundances of heavy isotopes.

Radioactivity was measured with a liquid scintillation counter using Optiphase HiSafe (Pharmacia, Uppsala, Sweden) as scintillation liquid.

RESULTS

The recovery of radioactivity, measured from the chloroform phase of the first extraction to the chloroform phase of the extraction following Lipidex-DEAP chromatography, was 76–99% for PtdIns4P and 58–88% for PtdIns(4,5) P_2 . H.p.t.l.c. of the material in these fractions showed that the PtdIns fraction and the PtdIns4P fraction contained additional material. PtdIns could be isolated by preparative h.p.t.l.c., whereas attempts to isolate PtdIns4P in this way were unsuccessful due to low recoveries and additional contamination. The major contaminant in the PtdIns4P fraction was slightly less polar than PtdIns4P. The PtdIns(4,5) P_2 fraction appeared to be pure on h.p.t.l.c.

The species composition was determined by f.a.b.m.s., as exemplified in Figs. 1–3. The compositions are given in Table 1. The most likely structures of the molecular species corresponding to a peak in the f.a.b. spectrum were deduced from the gas chromatographic analyses of fatty acid methyl esters obtained by trans-esterification and from previous results [2–4]. The variation in determined composition was clearly higher for PtdIns4P than for PtdIns(4,5) P_2 . This was probably due to the additional material present in the PtdIns4P fraction, giving peaks in the f.a.b. spectrum that could not be interpreted as being due to PtdIns4P species (Fig. 2). The predominant peak of the contaminating material appeared at m/z 863. Some peaks interfered with the determination of the species composition of PtdIns4P, especially the quantification of the dipalmitoyl species.

In the control rats, the composition of PtdIns4P was similar to that of PtdIns. The only significant differences were a higher proportion of the species containing a palmitoyl and a stearyl group ($P < 0.001$) and a lower proportion of the species containing a stearyl and a linoleoyl group ($P < 0.01$) in PtdIns4P than in PtdIns. In PtdIns(4,5) P_2 , the two completely saturated species were present in significantly ($P < 0.001$) higher (about twice) proportions than in PtdIns. In addition, PtdIns(4,5) P_2 had lower contents of the three arachidonoyl-containing species than PtdIns ($P < 0.001$ –0.05).

The results show highly significant differences between the ethanol-fed and control rats. Thus PtdIns species containing an arachidonoyl group were half as abundant in the ethanol-fed rats, while species containing a stearyl and no arachidonoyl group were twice as abundant in these animals. The percentage of PtdIns containing two palmitoyl residues was also lower in the ethanol-fed rats. No differences were seen for the species containing an oleoyl and a linoleoyl group, or for the species containing one palmitoyl and no arachidonoyl group. The differences in composition of PtdIns4P and PtdIns(4,5) P_2 between ethanol-fed and control rats were of the same type as for PtdIns, although less marked.

The acyl group composition was determined by gas chro-

Fig. 1. F.a.b.m.s. of PtdIns from the pancreas of an ethanol-fed rat (a) and a corresponding control rat (b)

The peaks are considered to represent the following acyl group compositions: 809, $\text{C}_{16:0} + \text{C}_{16:0}$; 837, $\text{C}_{16:0} + \text{C}_{18:0}$; 861, $\text{C}_{18:0} + \text{C}_{18:2}$; 863, $\text{C}_{18:0} + \text{C}_{18:1}$; 885, $\text{C}_{18:0} + \text{C}_{20:4}$.

Fig. 2. F.a.b.m.s. of PtdIns4P from pancreas of an ethanol-fed rat (a) and a corresponding control rat (b)

Fig. 3. F.a.b.m.s. of PtdIns(4,5)P₂ from pancreas of an ethanol-fed rat (a) and a corresponding control rat (b)

The peaks at *m/z* 863 and 923 are considered to be due to contaminants, and the following peaks are considered to represent PtdIns4P with the following acyl group compositions: 917, C_{16:0}+C_{18:0}; 941, C_{18:0}+C_{18:2}; 943, C_{18:0}+C_{18:1}; 965, C_{18:0}+C_{20:4}.

The peaks are considered to represent the following acyl group compositions: 969, C_{16:0}+C_{16:0}; 997, C_{16:0}+C_{18:0}; 1023, C_{18:0}+C_{18:1}; 1045, C_{18:0}+C_{20:4}.

Table 1. Molecular species composition of phosphoinositides from pancreas of rats fed an ethanol-containing liquid diet (E) and from the corresponding pair-fed controls (C), determined by f.a.b.m.s.

Assigned structures are based on molecular masses and most likely acyl groups as judged from previous results and gas chromatography after transesterification. Values are means ± s.d. (*n* = 4). Significance of differences: **P* < 0.05, †*P* < 0.01 and ‡*P* < 0.001 in comparisons of controls and ethanol-fed rats.

Acyl groups	PtdIns		PtdIns4P		PtdIns(4,5)P ₂	
	C	E	C	E	C	E
C _{16:0} +C _{16:0}	2.9±0.3	1.8±0.4†	9.6±5.5	8.8±5.1	6.0±0.5	3.2±0.7‡
C _{16:0} +C _{18:2}	3.5±0.4	2.9±0.4	4.1±1.8	4.3±1.6	3.2±0.3	2.8±0.5
C _{16:0} +C _{18:1}	7.3±0.5	7.0±0.4	8.0±0.5	6.6±0.9*	8.2±0.7	6.9±0.9
C _{16:0} +C _{18:0}	6.6±0.5	13.6±1.1‡	10.9±0.8	13.2±3.3	14.9±1.7	22.9±4.0*
C _{16:0} +C _{20:4}	6.0±0.1	2.4±0.4‡	5.8±0.7	5.5±1.6	4.5±0.3	2.6±0.7†
C _{18:1} +C _{18:2}	4.1±0.2	4.8±0.3*	4.2±0.6	5.2±0.5*	3.6±0.7	4.5±0.8
C _{18:0} +C _{18:2}	13.4±0.4	21.4±1.4‡	9.1±1.6	11.3±2.5	9.5±0.3	12.0±1.6*
C _{18:0} +C _{18:1}	10.4±0.7	23.3±3.1‡	8.7±1.9	14.5±2.3†	11.8±1.6	16.5±1.8†
C _{18:1} +C _{20:4}	7.8±0.3	3.7±0.4‡	8.0±0.8	7.0±0.2	6.7±0.5	5.3±1.1
C _{18:0} +C _{20:4}	37.9±1.9	19.2±1.3‡	31.6±5.3	23.6±4.8	31.5±2.1	23.2±3.6†

Table 2. Acyl group composition of phosphoinositides from pancreas of rats fed an ethanol-containing liquid diet (E) and from the corresponding pair-fed controls (C), determined by gas chromatography after transesterification to the methyl esters

Values are means $\pm$ S.D. ($n = 4$). Significance of differences: * $P < 0.05$, † $P < 0.01$ and ‡ $P < 0.001$ in comparisons of controls and ethanol-fed rats.

Acyl groups	PtdIns		PtdIns4P		PtdIns(4,5)P ₂	
	C	E	C	E	C	E
C _{16:0}	14.4 $\pm$ 0.6	11.6 $\pm$ 1.2†	16.9 $\pm$ 5.5	10.6 $\pm$ 2.1	12.3 $\pm$ 2.8	15.6 $\pm$ 1.5
C _{18:0}	41.6 $\pm$ 1.4	52.6 $\pm$ 3.8†	26.3 $\pm$ 8.0	20.4 $\pm$ 4.6	44.3 $\pm$ 3.1	48.9 $\pm$ 10.0
C _{18:1}	10.5 $\pm$ 9.5	14.7 $\pm$ 1.9†	12.4 $\pm$ 4.2	14.8 $\pm$ 6.8	10.5 $\pm$ 0.8	13.2 $\pm$ 5.9
C _{18:2}	7.4 $\pm$ 0.4	9.3 $\pm$ 1.1*	33.9 $\pm$ 17.9	48.2 $\pm$ 6.7	6.0 $\pm$ 0.6	9.0 $\pm$ 6.4
C _{20:4}	26.1 $\pm$ 1.6	11.8 $\pm$ 1.0‡	10.6 $\pm$ 2.5	6.1 $\pm$ 2.2*	26.9 $\pm$ 2.0	13.3 $\pm$ 3.0‡

matography of the fatty acid methyl esters. The results are given in Table 2, and show highly significant differences between the ethanol-fed and control animals. The content of arachidonoyl groups was more than 50 % lower in the ethanol-fed rats, and the content of stearoyl and linoleoyl groups was higher. The very high value for linoleoyl groups in PtdIns4P is considered to result from the contaminating material.

The total amounts of phosphoinositides per pancreas were calculated from the gas chromatographic analyses of the methyl esters without correction for losses. The tissue from the control rats yielded 162 ± 43 nmol per pancreas and that from the ethanol-fed rats contained 120 ± 22 nmol per pancreas. PtdIns4P and PtdIns(4,5)P₂ constituted similar fractions of the total, with mean values of 7–8 % (S.D. 3 %). The amount of arachidonoyl residues in PtdIns was significantly ($P < 0.05$) lower in the ethanol-treated rats (23 ± 8 nmol) than in the control rats (68 ± 23 nmol). This difference was more significant ($P < 0.001$) for PtdIns(4,5)P₂, where the amounts were 1.4 ± 0.3 nmol in the ethanol-fed rats and 4.4 ± 0.8 nmol in the control rats.

DISCUSSION

The amounts of PtdIns4P and PtdIns(4,5)P₂ in the rat pancreas were each about one-tenth of the amount of PtdIns. The stearoyl/arachidonoyl species predominated in all cases and there were no major differences in the composition of molecular species as determined by f.a.b.m.s. Thus there appears to be no marked selectivity in the phosphorylation steps in the PtdIns cycle, and the major diacylglycerol formed during stimulation can be expected to be the stearoyl/arachidonoyl species. A minor but significant difference was that the completely saturated species were about twice as abundant in PtdIns(4,5)P₂ as in PtdIns. This might have relevance for the effects of the former, since protein kinase C shows some specificity for the acyl group composition of the diacylglycerol [13].

The differences in PtdIns composition between ethanol-fed and control rats were of the same type as previously observed by analysis of the diacylglycerols released by phospholipase C [1,4]. Thus the abundance of arachidonoyl-containing species in the ethanol-treated rats was about half that in the controls. The quantitative analysis showed an even more pronounced difference in the absolute amount of arachidonoyl groups in PtdIns. The reason for this effect of ethanol is not known. The possibility that

peracetic acid formed from ethanol might inhibit arachidonic acid incorporation [14] would not explain why other phospholipids were unaffected [1,4].

The ethanol-induced change in PtdIns composition is much more marked in pancreas and submaxillary glands than in liver [1,3,15], and thus appears to be selective for exocrine glands [3], where the PtdIns cycle is very active. The changes in PtdIns4P and PtdIns(4,5)P₂ were similar to those in PtdIns, although the decrease in arachidonoyl-containing species was less marked. Thus the change does not appear to be due to increased hydrolysis of arachidonoyl-containing PtdIns(4,5)P₂ by phospholipase C. Instead, it may be suggested that ethanol interferes with the compartmentation of diacylglycerol and phosphatidic acid in the PtdIns cycle, causing dilution by other lipids containing less arachidonate [3].

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REFERENCES

1. Cronholm, T. & Curstedt, T. (1984) *Biochem. Pharmacol.* **33**, 1105–1109
2. Cronholm, T. & Curstedt, T. (1985) *Alcohol* **2**, 183–186
3. Cronholm, T. & Curstedt, T. (1986) *Biochem. Pharmacol.* **35**, 1608–1611
4. Cronholm, T., Neri, A., Karpe, F. & Curstedt, T. (1988) *Lipids* **23**, 841–846
5. Low, M. G. & Saltiel, A. R. (1988) *Science* **239**, 268–275
6. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
7. Sjövall, J., Raftar, J., Larsen, G. & Egestad, B. (1983) *J. Chromatogr.* **276**, 150–156
8. DeCarli, L. M. & Lieber, C. S. (1967) *J. Nutr.* **91**, 331–336
9. Lieber, C. S. & DeCarli, L. M. (1989) *Alcohol Alcohol.* **24**, 197–211
10. Schacht, J. (1981) *Methods. Enzymol.* **72**, 626–631
11. Renkonen, O. & Luukonen, A. (1976) in *Lipid Chromatographic Analysis*, 2nd edition, vol. 1 (Marinetti, G. V., ed.), pp. 1–58, Marcel Dekker, New York
12. Medh, J. D. & Weigel, P. H. (1989) *J. Lipid Res.* **30**, 761–764
13. Kerr, D. E., Kissinger, L. F., Gentry, L. E., Purchio, A. F. & Shoyab, M. (1987) *Biochem. Biophys. Res. Commun.* **148**, 776–782
14. Allen, D. W., Newman, L. M. & Okazaki, I. J. (1991) *Biochim. Biophys. Acta* **1081**, 267–273
15. Ellingson, J. S., Janes, N., Taraschi, T. F. & Rubin, E. (1991) *Biochim. Biophys. Acta* **1062**, 199–205