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PHOSPHOLIPASE D MEDIATED TRANSPHOSPATIDYLATION AS A POSSIBLE NEW PATHWAY OF ETHANOL METABOLISM IN ISOLATED RAT PANCREATIC ACINI *

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Activation of pancreatic phospholipase D (PLD) has been previously observed in response to caerulein (Cae), phorbol myristate acetate and growth factors. Although PLD involvement has been postulated in pancreatic cell proliferation and differentiation, the physiological role of this enzyme in pancreatic cells still remains unclear. In the presence of ethanol, PLD catalysed transphosphatidylation reaction, forming phosphatidylethanol (PEt). This study was thus undertaken to determine the involvement of PLD in ethanol metabolism in isolated pancreatic acini and to show the potential physiological consequences of transphosphatidylation. Dispersed pancreatic acini prelabelled with ^{3}H myristic acid were incubated with 500 pM Cae in the presence or absence of different concentrations of ethanol, and labelled phosphatidylethanol (^{3}H PEt) production or phosphatidic acid (^{3}H PA) accumulation were measured. The production of PEt after Cae stimulation in pancreatic acini was significant from 0.5% up to 4% of ethanol in the medium and was not dependent on increasing concentration of ethanol. Prolonged up to 2 h stimulation with Cae in the presence of 1% ethanol did not increase PEt production which was almost stable since 5 min of stimulation. In the presence of different concentrations of ethanol (1—4%), the significant inhibition of PA accumulation was obtained after Cae stimulation, similar to inhibition with a specific PLD inhibitor — wortmannin. These data indicate that Cae activated PLD in the presence of ethanol caused PEt producttion in pancreatic acini. During formation of PEt in pancreatic acinar cells significant and parallel inhibition of PA accumulation was observed. This indicates the relation of PLD activation in isolated pancreatic acini to ethanol metabolism. Ethanol can act as an inhibitor of PLD activity. Since PA, a product of PLD, is known as a second messenger probably involved in cell proliferation and differentiation, this may suggest a potentially new mechanism for pancreatic tissue injury after ethanol ingestion.

Key words: *phospholipase D, transphosphatidylation, ethanol, phosphatidylethanol, phosphatidic acid inhibition*

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INTRODUCTION

There is increasing evidence that phosphatidylcholine (PC) metabolism undergoes signal-dependent changes, leading to the formation of intracellular messenger molecules such as diacylglycerol and phosphatidic acid (PA) (1—3). In most cell types both, phospholipase C and phospholipase D (PLD) activities may be involved in PC breakdown (4, 5). Recently we have demonstrated the evidence for phospholipase D activity in isolated rat pancreatic acini (6). This pathway can be selectively activated by CCK, the most potent trophic factor to the pancreas, through the occupation of the high-affinity CCK_A receptor, but not by carbachol (7). Phorbol myristate acetate and growth factors are also known to activate PLD in pancreatic acini (8, 9). In the presence of water PLD hydrolyses phosphatidylcholine, yielding phosphatidic acid and choline. In the presence of primary alcohol, however, the preferred reaction will be that of transphosphatidylation, yielding phosphatidylethanol (PEt). This reaction is a unique feature of PLD and being PLD specific, becomes the marker of choice for the enzyme's measurement (4, 5). The physiological function of PLD remains still unknown. Phosphatidic acid produced from phosphatidylcholine hydrolysis by PLD has been recognised as a mitogenic agent in may cell systems (10, 11). Regarding to the pancreas, significant activation of PLD in response to growth associated processes, stimulated by pancreatic juice diversion, pancreatectomy (12) or during regeneration after caerulein — induced acute pancreatitis (13), was demonstrated, and the involvement of PLD in pancreatic growth and regeneration was thus postulated. Yet, the physiological significance of transphosphatidylation is still unclear. The formation of phosphatidylethanol in the brain was postulated to be involved in intoxication by and tolerance to ethanol (14). The exact mechanism of action of ethanol in many tissue is still unknown. Presently the most accepted view is that ethanol exerts its pharmacological effects on the lipids of the cell membrane and causes an increased fluidity and alteration in the integral membrane proteins (15, 16). In view of all those information's suggesting the possibility of ethanol metabolism in pancreas via PLD, we sought to investigate the influence of ethanol on PLD activity *in vitro* in isolated pancreatic acini, measured as PEt and PA and to determine the physiological consequences of PEt production.

MATERIALS AND METHODS

MATERIALS

Bovine serum albumin (BSA; fraction V and fatty acid free), soybean trypsin inhibitor type 2-S (SBTI), N-2-hydroxyethyl piperazine-N^1-2-ethane sulphonic acid (HEPES), standards and solvents for thin layer chromatography (TLC) and for nucleic acid determination were purchased from

Sigma. Purified collagenase (1 424 units/mg) was from Worthington Biochemicals. Silica gel G TLC plates (LK6D) were obtained from Whatman. [^{3}H] myristic acid (56 Ci/mmol) was from Du Pont. Caerulein was from Farmitalia Carlo Erba and phosphatidylethanol from Avanti Polar Lipids. Wortmannin was from Sandoz.

Male Wistar rats were used for the study (200—220 g) (n = 60).

METHODS

Preparation of pancreatic acini

Acini were prepared as reported by Peikin et al. (17) from a 300 mg piece of pancreatic tissue. Acini from 5 pancreas were pooled and resuspended in 32 ml of an enriched HEPES buffered solution [(in mM) 24.1 HEPES, 98 NaCl, 6 KCl, 2.1 KH_2PO_4, 0.5 $CaCl_2$, 1.2 $MgCl_2$, 5 sodium pyruvate, 5 sodium fumarate, 5 sodium glutamate, 11.4 glucose and 0.1% (w/v) SBTI, 0.5% (w/v) BSA fatty acid free, adjusted to pH 7.4].

PLD activity

PLD activity was measured by determination of the PA and/or PEt formation from labelled phosphatidylcholine (PC), and was described elsewhere (6). Briefly, for PA determination after 1 h of incubation with 5 μCi/ml of [^{3}H] myristic acid, the 5 ml of acini in each flask were washed twice and resuspended in freshly oxygenated medium containing 200 μM of propranolol, to measure PC hydrolysis in response to caerulein for the indicated time period tn the presence and absence of ethanol. At the end of each time period, 1 ml of acini was removed and quickly centrifuged at 10,000 g in a microcentrifuge for 15 s. The supernatant was removed, the pellet was washed with incubation medium and then 2 ml of methanol: 10 mM glycine (5:2 vol./vol.) was added and cells were detached mechanically with a spatula. To this methanol: glycine mixture, 1 ml of chloroform was added and mixed; 1 ml of chloroform and 1 ml of water were then added to this mix, and the phases were separated by a 5-min centrifugation at 1.000 g. Radioactivity present in the chloroform phase was determined. The samples of the chloroform phase with standards added were dried under a stream of nitrogen and redissolved in 50 ml of chloroform. PA was separated in a solvent system contining chloroform: acetone: methanol: acetic acid: water (50:20:15:10:5, vol./vol.) (18). After separation and exposure to iodine vapour, the area containing PA was scraped, and radioactivity was counted. Radioactivity in PA was expressed as a percent of total radioactivity in chloroform phase.

For determination of phosphatidylethanol production acini were also incubated with ^{3}H myristic acid, then washed and incubated for the indicated time with or without caerulein, in the medium containing different concentrations of ethanol. Chloroform and aqueous phases were separated by the procedures described above. Standard samples of PEt were added to chloroform phases, and these phases were dried under nitrogen and spotted on TLC plates. The plates were developed in a system containing chloroform: methanol: ammonium hydroxide (65:30:3, vol./vol.) (19). Areas containing PEt were scraped, and radioactivity was caunted. PEt was normalised to the percent of total amount of the labelled lipids. For some experiments wortmannin was used to inhibit PLD activity.

Statistical analysis

Results were analysed by one-way analysis of wariance followed by Fisher test. Results were considered significantly different at $p < 0.05$.

RESULTS

PLD activity in isolated pancreatic acini measured as PEt formation in the presence of different ethanol concentrations

Our first approach was to determine PEt production after Cae stimulation (500 pM) with different ethanol concentrations (from 0.25% up to 2%). As shown in *Fig. 1*, the PEt production in response to caerulein, in maximal dose for PLD activation, became significant from 0.5% ethanol into the medium up to 2% and was not dependent on increasing ethanol concentration. 0.25% of ethanol in the medium was not enough to increase significantly PEt production.

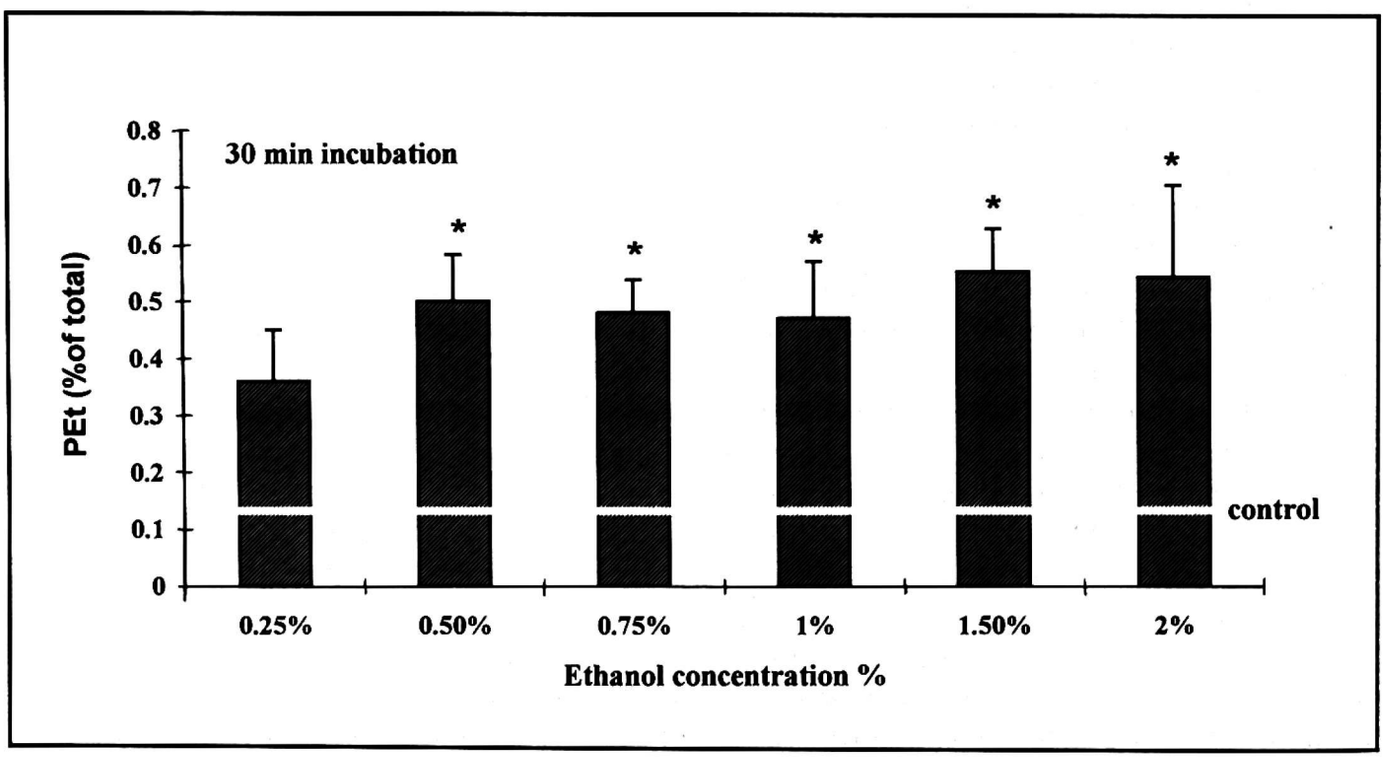

Fig. 1. Cae stimulated phospholipase D activity with different ethanol concentrations. Pancreatic acini were stimulated with Cae in the presence of different ethanol concentrations (from 0.25 up to 2%) of ethanol for 30 min. PEt production was measured as described in methods. Results are the means ± SE from 4 separated experiments made in duplicate. * significantly different from Cae stimulation without ethanol (control) ($p < 0.05$)

Time course of PA accumulation in isolated pancreatic acini after Cae stimulation in the presence of different ethanol concentrations (from 0.5 up to 4%)

The next step was to determine the influence of different ethanol concentration, optimal for PEt formation, on PA accumulation. As shown in *Fig. 2* we observed significant inhibition of PA accumulation in the presence of different ethanol concentrations. This inhibition was significant after 15 and 30 min of incubation for 0.5% and 0.8% of ethanol and at each time point examined for 1, 2, and 4% of ethanol. Inhibition of PA accumulation was parallel to PEt production, what can suggest than ethanol acts as a competitive inhibitor of PLD activity. *Fig 3* shows similar and parallel pattern of changes in PA and PEt at the same time point, when expressed as a percent of control values. This suggests that PA measured in our study cames mainly from PLD activity and its production could be inhibited by different ethanol concentrations.

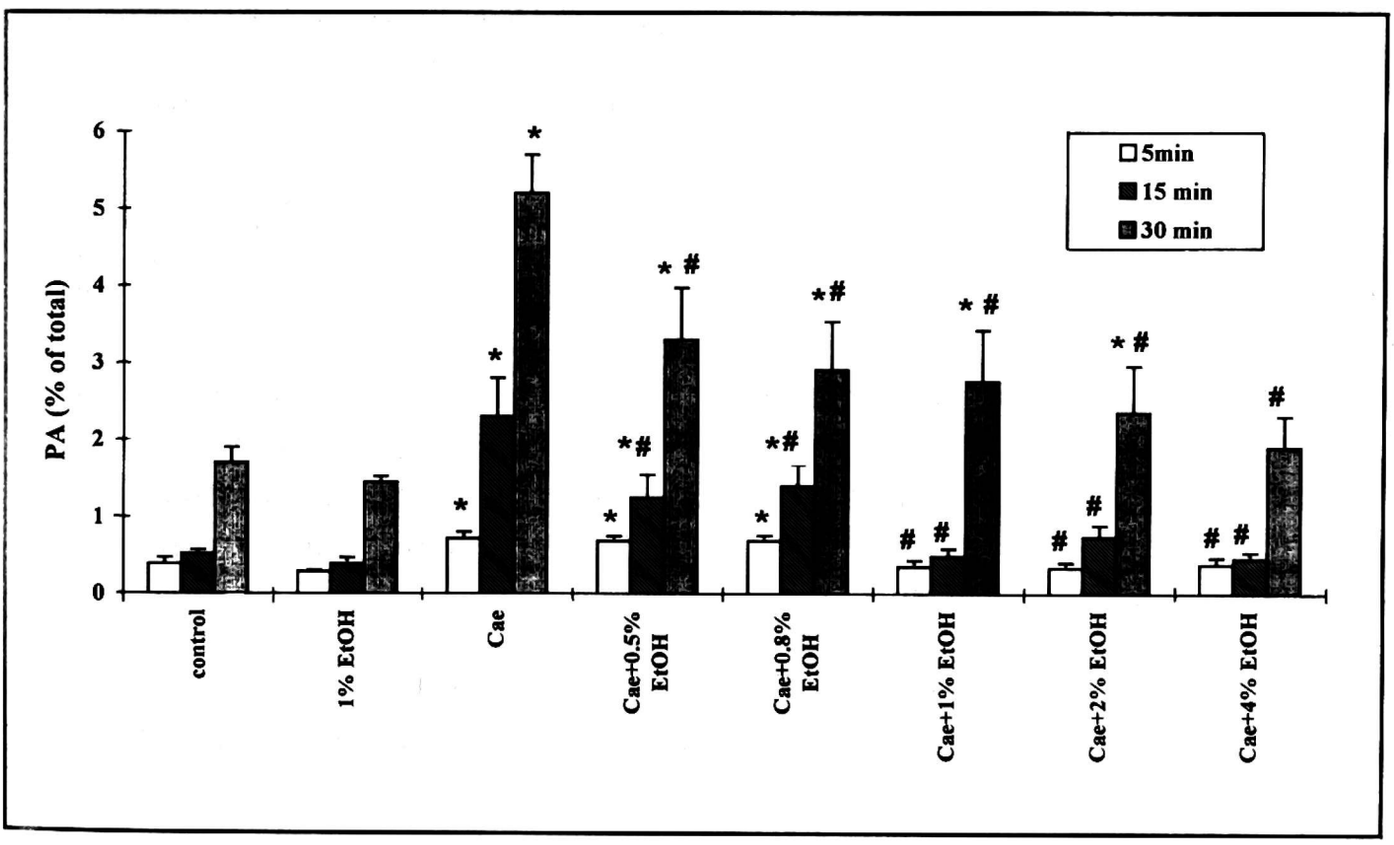

Fig. 2. The influence of different ethanol concentration on Cae stimulated phosphatidic acid accumulation in isolated pancreatic acini. Pancreatic acini were stimulated with 500 pM Cae in the presence or absence of ethanol (EtOH) (0,5, 0,8 1, 2 and 4% respectively), for the indicated time periods. To favour PA accumulation 200 mM propranolol was used. The reaction was stopped, lipids were extracted and PA was separated and counted as described in methods. Results are the means ± SE of 4 separated experiments made in duplicates. # significant difference between Cae and Cae with ethanol ($p < 0.05$). * significant difference between control and Cae stimulated acini ($p < 0.05$).

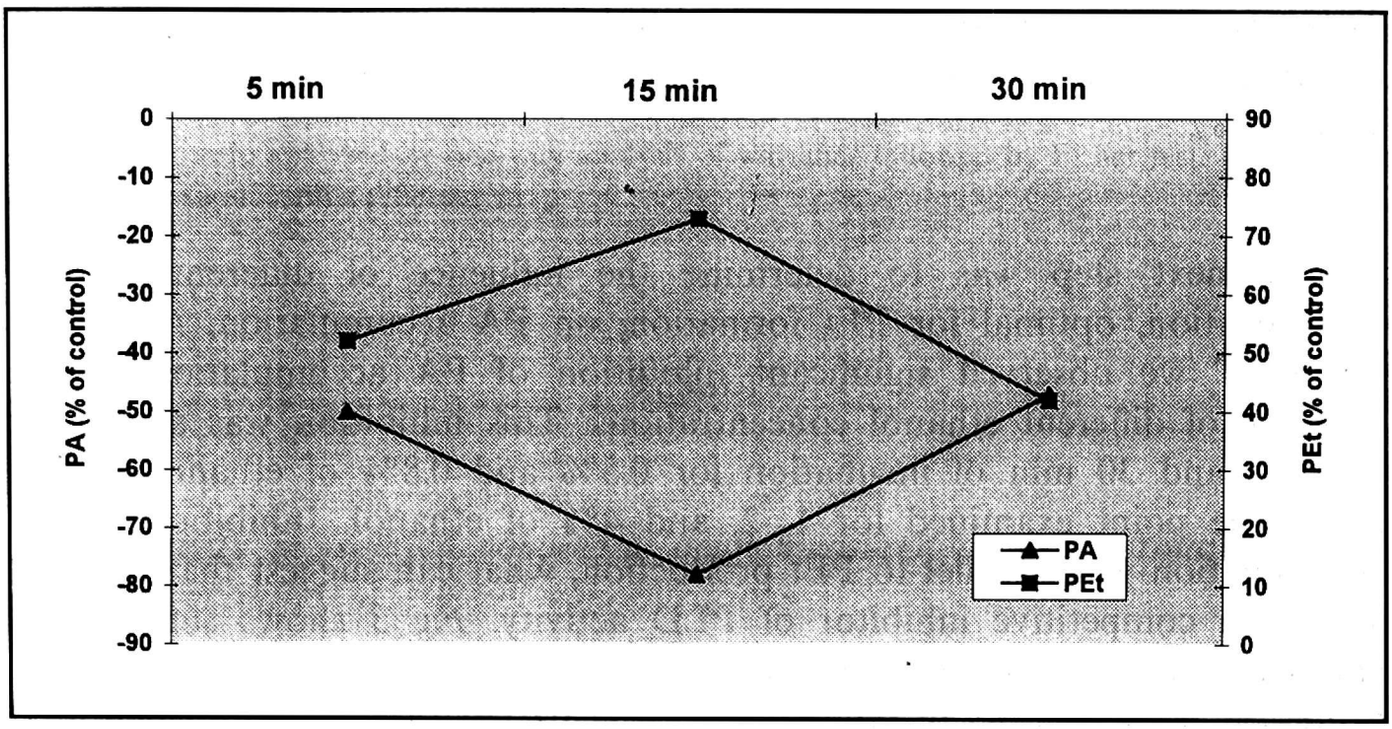

Fig. 3. Parallelism in PA inhibition and PEt production in pancreatic acini. Pancreatic acini were stimulated with 500 pM Cae in the presence or absence of 1% of ethanol for the indicated time periods. In some experiments PEt was measured and in others PA accumulation in the presence of propranolol was established. The reaction was stopped, lipids were extracted, PA and PEt were separated and counted as described in methods. PA and PEt were expressed as a percent of control. Results are the means ± SE of 4 separated experiments made in duplicates.

The comparison between inhibition of PLD, measured as PA production, by wortmannin, a specific enzyme inhibitor, and 1% of ethanol in isolated rat pancreatic acini

In the next step was compared the influence of ethanol and wortmannin — a specific PLD inhibitor, as shown in *Fig. 4.* 1% of ethanol was almost as potent in inhibiting of PA accumulation as maximal dosis of wortmannin (50 nM). This dosis was able to inhibit totally PEt production and tyrosine kinase activity, stimulated by Cae (24).

The influence of prolonged Cae stimulation on PEt accumulation in isolated rat pancreatic acini

According to literature, phosphatidylethanol is metabolically stable. In the next part of our stady we tried to determine if the accumulation of PEt in the cells could be observed after prolonged up to 2 h stimulation with Cae in the presence of optimal ethanol concentration (1%). As shown in *Fig. 5* we did not observe accumulation of PEt, since its production was significant after 5 min and did not change in time. This cam suggest that PEt could be metabolised in pancreatic acini.

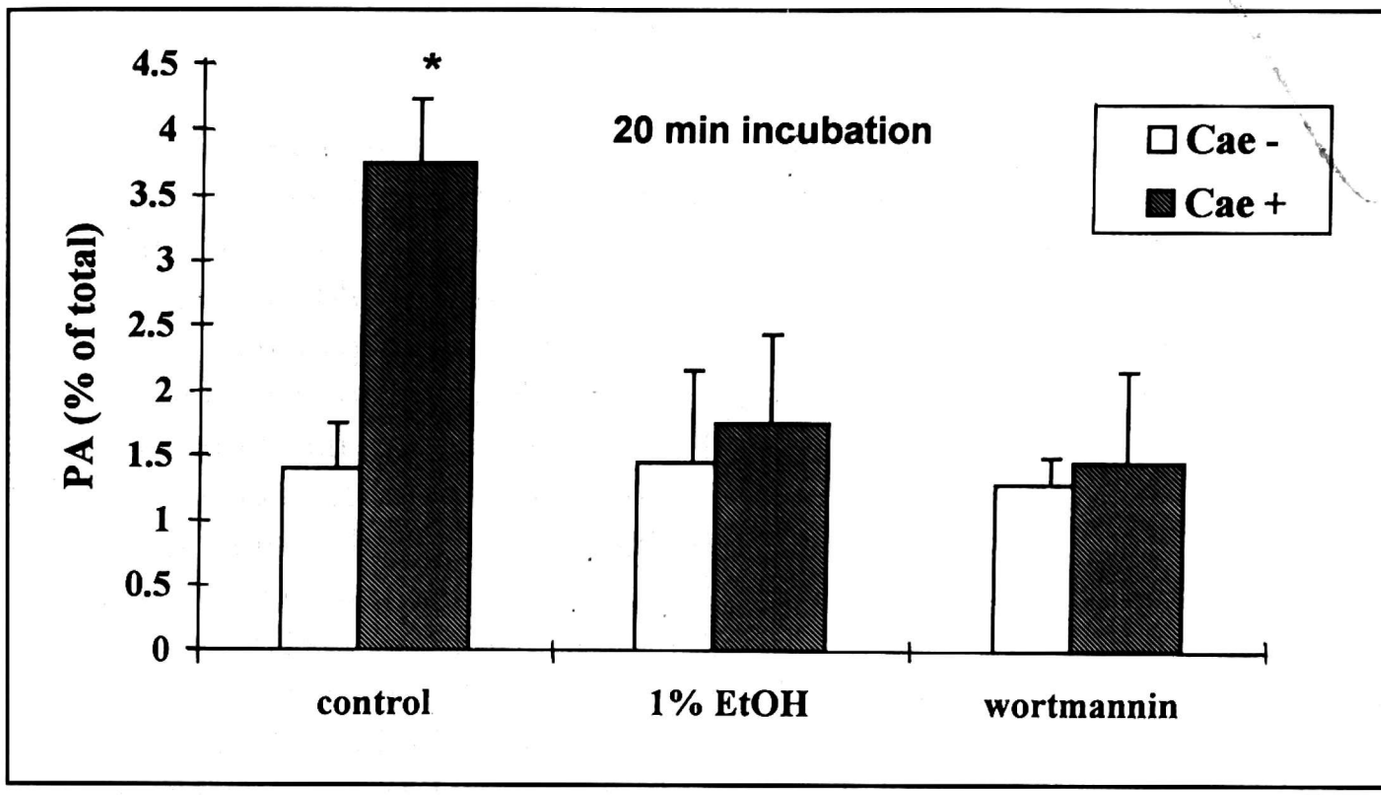

Fig. 4. Comparison of inhibitory effect of 1% ethanol and 50 nM wortmannin. Pancreatic acini were incubated with or without 500 pM Cae in the presence of ethanol (1%) or wortmannin (50 nM) for 20 min. PA accumulation in the presence of propranolol was established. The reaction was stopped, lipids were extracted, PA was separated and counted as described in methods. Results are the means ± SE of 4 separated experiments made in duplicates. * significantly different from acini without Cae stimulation ($p < 0.05$)

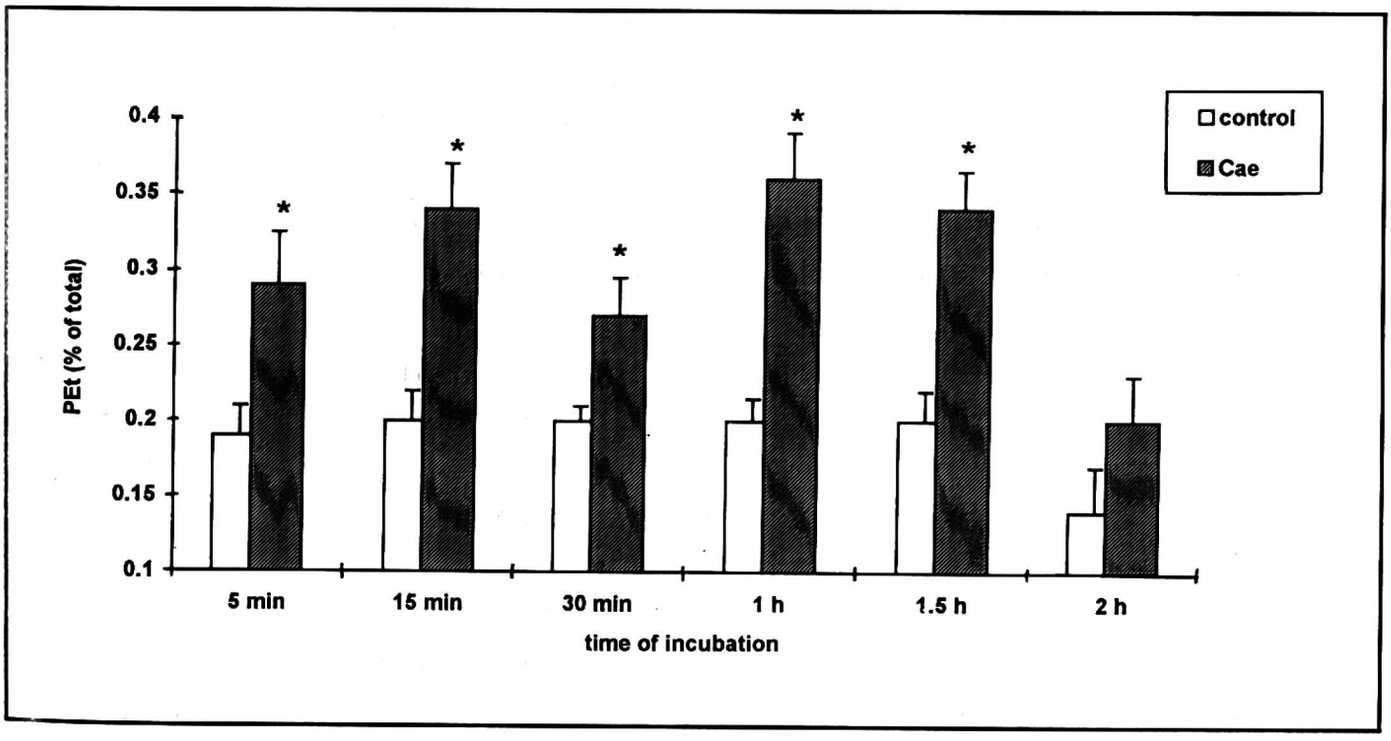

Fig. 5. Prolonged incubation of pancreatic acini with Cae in the presence of 1% ethanol. Pancreatic acini were stimulated with 500 pM Cae in the presence of ethanol (1%) from 5 min up to 2 h. PEt production was then established as described in methods. Results are the means ± SE of 4 separated experiments made in duplicates. * significantly different from control ($p < 0.05$)

DISCUSSION

Formation of PEt in dispersed acini in response to Cae (6), PMA or growth factor but not to carbachol (8, 9) was recently demonstrated in the presence of ethanol in the medium. Thus the existence of a potential new pathway for ethanol metabolism in pancreatic tissue could be suggested. In present paper we confirm significant PEt production in pancreatic acini not dependent on ethanol concentration, from 0.5% up to 4%. Our results indicate also that ethanol (0,5—4%) could act as an inihibitor of PLD, inhibiting PA accumulation; in the same time parallel increase in PEt production was observed. This suggests that PA measured in our experiments comes mostly from PLD activity and that in the presence of ethanol, physiological PLD activity (PA production) is inhibited. The physiological consequences of transphosphatidylation in many cell systems are still unknown (3—5). Since ethanol in pancreas is a known aethiologic factor of acute and chronic pancreatitis and pancreatic tissue injury (20—22) this can suggest potential involvements of transphosphatidylation and its consequences in this process. Ethanol diminished PA production by PLD and caused its replacement by PEt. We can thus speculate that transphosphatidylation is a novel way to „selectively" able increments in PA from pancreatic membranes and thereby be potential new mechanism for membranes injury. In some other cells, production of PEt from PC leads to an inhibition of regulated secretory events (23). In the pancreas, the association of PA production with secretion does not seem to exist since wortmannin, a PLD inhibitor, did not inhibit Cae induced amylase secretion (24). Furthermore, concentrations of ethanol, which are associated with PEt production do not inhibit secretory responses and were even described as a weak stimulus of amylase release, probably by PLC activation (25). The influence on amylase release is still controversial, since recently Perkins et all shown increase in all digestive enzyme secretion exept amylase. Amylase synthesis rate and amylase mRNA level decreased in the pancreas of rats treated with ethanol (26). It is interesting that in our study ethanol inhibited PA accumulation in the same manner as maximal dosis of specific PLD inhibitor-wortmannin. Wortmannin can inhibit tyrosine kinase activity in isolated rat pancreatic acini, and also it is known to inhibit growth of AR4 2J cell, with concomitant inhibition of PLD and tyrosine kinase (24, 27).

On the other hand phosphatidylethanol seems to be metabolically stable in may cells and could be thus accumulated in chronic alcoholism to the level, which is detrimental to pancreatic cell function. Our pilot study in this field does not confirmed the PEt accumulation, since prolonged incubation of pancreatic acini in the presence of ethanol did not increase PEt content in pancreatic acini. PEt level was almost stable from 5 min up to 1.5 h of

incubation with Cae. This could suggest that PLD activity is already completed after 15 min or can serve as an evidence for PEt metabolism in pancreatic acini. Our experiment does not exclude the possibility that PEt in the cells could change cell function, membranes permeability and has some influence on pancreatic tissue injury. According to earlier observations, ethanol in the concentrations 180 mM, (sufficient for PEt production), does not change pancreatic acini viability and permeability itself, but facilitate the increased permeability together with hypoxia (28) or PLA_2 (29). In pancreatic iselts of diabetic patients the increase of phosphatidylglycerol and phosphatidylsorbitol content (product of transphosphatidylation in chronic hyperglycaemia) in membranes are found and the involvement of these changes in insulin secretion and action is suggested (30). Further studies are needed to establishe the role of transphosphatidylation in pancreatic cell with regards to ethanol consumption.

On the other hand PA is known as a mitogenic agent in many cell types and seems to be involved as a second messenger in some cellular responses including cell proliferation and regeneration (10, 11). Regarding to pancreas, PLD activation does not seem to be involved in secretion, but rather in pancreatic cell growth and regeneration. We observed significant accumulation of PA coming from PLD after caerulein, PMA and growth factor and in growth associated processes as pancreatic juice diversion, subtotal pancreatectomy, Cae infusion in trophic dosis and during regeneration period after acute pancreatitis (7, 8, 12, 13). Therefore, it is intriguing to speculate, if reduction of PA accumulation, showed in our study contributes to ethanol — induced impairment of pancreatic regeneration, especially since ethanol concentration achievable *in vivo* in humans (0.1—0.6%) provide sufficient acceptor to support transphosphatidylation (from 0.5 to 2%). However recent studies on the pancreatic growth after chronic ethanol treatment are controversial. Pap and associates reported that ethanol counteracted the trophic pancreatic effect of endogenous cholecystokinin on partially resected adults rat pancreas (31). Some other authors does not confirmed this effect, since ethanol given together with camostate did not affect trophic effect of the trypsin inhibitor (32).

In summary our present data indicate the possibility of ethanol metabolism in pancreas and potential new pathway of pancreatic injury after ethanol ingestion. Further study are needed to confirm this hypothesis and to show the influence of *in vivo* ethanol ingestion on PLD in pancreas.

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REFERENCES

1. Shukla SD, Halenda SP. Phospholipase D in cell signilling and its relationship to phospholipase C. *Life Sci* 1991; 48: 851—866.
2. Huang C, Wyke RL, Daniel LW, Cabot MC. Identification of phosphatidylcholine — selective and phosphatidylinositol selective phospholipase D in Madine-Darby canine kidney cells. *J Biol Chem* 1992; 267: 16859—16865.
3. Billah MM. Phospholipase D and cell signalling. *Current Opinion Immunol* 1993; 5: 114—123.
4. Thompson NT, Bonser RW, Garland LG. Receptor coupled phospholipase D and its inhibition. *Trends Pharmacol Sci* 1991; 12: 404—408.
5. Billah MM, Anthes JC. The regulation and cellular function of phosphatidylcholine hydrolysis. *Biochem J* 1990; 269: 281—291.
6. Rydzewska G, Rossignol M, Morisset J. Involvement of phospholipase D in caerulein - induced phosphatidylcholine hydrolysis in rat pancreatic acini. *Am J Physiol* 1993; 265: G725—G734.
7. Rivard N, Rydzewska G, Lods J-S, Martinez J, Morisset J. Pancreas growth, tyrosine kinase, PtdIns 3-kinase, and PLD involve high-affinity CCK-receptor occupation. *Am J Physiol* 1994; 266: G62—G70.
8. Rydzewska G, Morisset J. Specificity of phospholipase D activation by cholecystokinin and phorbol myristate acetate but not by carbamylcholine and A 23187 in rat pancreatic acini. *Digestion* 1995; 56: 127—136.
9. Rydzewska G, Morisset J. Activation of pancreatic acinar cell phospholipase D by epidermal, insulin-like, and basic fibroblast growth factors involves tyrosine kinase. *Pancreas* 1995; 10: 59—65.
10. Moolenar WE, Kruijer W, Tiley BC, Verlaan J, Bierman AJ, de Latt SW. Growth-factor-like action of phosphatidic acid. *Nature* 1986; 323: 171—173.
11. Putney JW, Weiss SJ, Val De Walle CM, Haddas RA. Is phosphatidic acid a calcium ionophore under neurohormonal control? *Nature* 1980; 284: 345—347.
12. Rivard N, Rydzewska G, Boucher C, Lods J-S, Cavlo E, Morisset J. Cholecystokinin activation of tyrosine kinases. PTdInositol 3-kinase and phospholipase D: a role in pancreas growth induction? *Endocrine J* 1994; 2: 393—401.
13. Rydzewska G, Morisset J. Dynamics of phospholipase D and tyrosine kinase activaties during caerulein - induced acute pancreatitis in rats. *Pancreas* 1995; 10: 382—388.
14. Kanfer JN, Hattori H, Orihel D. Reduced phospholipase D activity in brain tissue samples from Alzheimer's disease patients. *Ann Neurol* 1986; 20: 265—267.
15. Chapman BA, Pattinson NR. The effect of ethanol on phospholipid metabolism in rat pancreas. *Biochem Pharmacol* 1988; 37: 1897—1902.
16. Sommer JB, Coley PW, Pirola RC. Influence of ethanol on pancreatic lipid metabolism. *Exp Mol Pathol* 1980; 33: 231—239.
17. Peikin SR, Rothman AJ, Batzri S, Gardner JD. Kinetics of amylase release by dispersed acini from guinea pig pancreas. *Am J Physiol* 1978; 235: E743—E749.
18. Matozaki T, Goke B, Tsunoda Y, Rodrigez M, Martinez J, Williams JA. Two functionally distinct cholecystokinin receptors show different modes of action on Ca^{2+} mobilisation and phospholipid hydrolysis in isolated rat pancreatic acini. Studies using a new cholecystokinin analogue, JMV-180. *J Biol Chem* 1990; 265: 6247—6254.
19. Huang C, Cabot MC. Vasopressin-induced phosphoinositide and phosphatidylcholine degradation in fibroblast. Temporal relationship for formation of phospholipase C and phospholipase D product. *J Biol Chem* 1990; 265: 17468—17473.
20. Di Magno EP, Malagelada JR, Go VL. Relationship between alcoholism and pancreatic insufficiency *Ann NY Acad Sci* 1975; 252: 200—207.

21. Wilson JS, Korsten MA, Pirola RC. Alcohol induced pancreatic injury (part 2). Evolution of pathogenic theories. *Int J Pancreatol* 1989; 4: 233—250.
22. Singh M, Simsek H. Ethanol and the pancreass. Current status. *Gastroenterology* 1990; 98: 1051—1062.
23. Chabot MC, Mc Phail LC, Wykle RL, Kennerly DA, Mc Call ChE. Comparison of diglyceride production from choline — containing phosphoglycerides in human neutrophils stimulated with N-formylmethionyl-leucylphenyloalanine, ionophore A23187 or phorbol 12-myristate 13-acetate. *Biochem J* 1992; 286: 693—699.
24. Rivard N, Rydzewska G, Lods J-S, Morisset J. Novel model in integration of signalling pathways in rat pancreatic acinar cells. *Amer J Physiol* 1995; 269: G352—G362.
25. Ponnappa BC, Hoek JB, Waring AJ, Rubin E. Effect of ethanol on amylase secretion and cellular calcium homeostasis in pancreatic acini from normal and ethanol — fed rats. *Biochem Pharmacol* 1987; 36: 69—70.
26. Perkins PS, Rutherford RE, Pandol SJ. Effect of chronic ethanol feeding on digestive enzyme synthesis and mRNA content in rat pancreas. *Pancreas* 1995; 10: 14—21.
27. Morisset J, Douziech N, Rydzewska G et al. Cell signalling pathway involved in PACAP - induced AR4-2J cell proliferation. *Cellular Signalling* 1995; 7: 195—205.
28. Siech M, Letho G. Influence of ethanol on survival of acinar cells isolated from rat pancreas. Res Exp Med 1992; 192: 57—63.
29. Letko G, Siech M. Effect of phospholipase A_2 and ethanol on the survival of acinar cells isolated from the rat pancreas. *Digestion* 1992; 51: 193—198.
30. Metz SA, Dunlop M. Production of phosphatidylethanol by phospholipase D phosphatidyl transferase in intact or dispersed pancreatic islets: evidence for the in situ metabolism of phosphatidylethanol. *Arch Biochem Bioph* 1990; 283: 417—428.
31. Pap A, Nagy I, Takacs T, Hajnal F, Toth D, Varro V. Mechanism of action of alcohol administration on the tropic effect of soyben inhibitor and cholecystokinin octapeptide in rat. *Int J Pancreatol* 1989; 5: 263—272.
32. Kisfalvi K, Varga G, HajnaL F, Friess H, Buchler M. Alcohol does not affect the trophic pancreatic effect of CCK and its plasma level in young rats. *Pancreas* 1994; 9: 399—401.

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