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Biochemical Pharmacology, Vol. 35, No. 9, pp. 1608–1611, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

Effects of ethanol and secretagogues on the composition of phosphatidylinositol in pancreas and submaxillary gland

(*Received* 13 *June* 1985; *accepted* 4 *November* 1985)

Acute and chronic pancreatitis are severe complications of alcoholism, but the pathogenesis is incompletely understood [1]. Rats fed an ethanol-containing diet for 24 days had a lower content of arachidonoyl-containing phosphatidylinositols in pancreas and submaxillary gland than the pair-fed controls [2, 3].This might reflect changes in the stimulus-secretion coupling, since phosphatidylinositols or polyphosphoinositides are involved in this process [4–8]. No effect of acute ethanol administration was found in the previous study [2], and the time-course of the appearance and disappearance of the change was therefore investigated both in pancreas and submaxillary gland. Since the changes observed in both organs might be due to chronic hyperstimulation, this was simulated by administration of secretagogues.

Materials and methods

Male Sprague–Dawley rats were used. Rats weighing about 100 g were fed a liquid diet for 24 days [9]. The alcohol diet, which provides 36% of the energy and the control diet with carbohydrate replacing ethanol, were obtained from Bioserv, Inc. (Frenchtown, NJ). Alcohol was introduced stepwise, and the controls were pair-fed [9] also when control diet was substituted for ethanol diet for 3 days after the 24 days of ethanol administration. In another experiment the liquid diet was given to 170 g rats for 6 days followed by one day of starvation. Rats weighing about 200 g were used in the other experiments.

Intravenous infusion (1 ml/hr) of solutions in saline (9 g/l) were performed in the femoral vein with a thin polyethylene catheter. Caerulein (Sigma Chemical Co.) was infused for 18 hr at a rate of 50 ng/hr, known to cause maximal stimulation of the pancreas [10]. Carbamyl-β-methylcholine (Sigma Chemical Co.) was infused for 18 hr or 4 days at a rate of 150 μg/hr that causes significant stimulation of pancreatic protein secretion [11]. The rats were kept in restraining cages with free access to water, and, in the 4-day experiments, free access to food pellets.

The rats were killed by cervical dislocation, and the pancreas and submaxillary gland were taken out, avoiding visible fat. The phosphatidylinositols were isolated and hydrolysed, and the diacylglycerols were analyzed as trimethylsilyl ethers as previously described [2, 3].

Student's *t*-test was used for statistical analysis.

Results

Identification of phosphatidylinositol species. The trimethylsilyl ethers of the diacylglycerols obtained from the phosphatidylinositols of pancreas and submaxillary gland were not completely separated by the gas chromatographic method. The major components in the gas chromatographic peaks were identified by gas chromatography/mass spectrometry (Table 1). It is assumed that the more unsaturated acyl group was in the 2-position.

Effects of ethanol. Rats fed an ethanol-containing liquid diet for 24 days had not the same phosphatidylinositol composition in the submaxillary gland as the corresponding pair-fed controls (Table 1). Thus, the arachidonoyl-containing species (36:4 and 38:4) were significantly less abundant whereas more saturated di-C_{18}-acyl-containing species contributed to a larger extent in the ethanol-fed animals. The fractions of both di-C_{16}-containing species were lower in the ethanol-fed rats. Differences in the same direction but smaller and less significant were seen when the diets were given for only 6 days followed by a day of starvation (Table 2). The difference between ethanol-fed and control groups was only slightly decreased by feeding control diets to both groups for three days after 24 days of pair-feeding the test diets.

Differences were also seen in the phosphatidylinositol composition in pancreas of rats pair-fed the ethanol-containing and control diets for 6 days followed by a day of

Table 1. Composition of phosphatidylinositols in submaxillary gland of rats fed diets with or without ethanol for 24 days

Acyl carbons: double bonds	Acyl groups in major molecular species	Percentage composition Control (N = 5)	Ethanol (N = 5)
32:1	1-Palmitoyl-2-palmitoleoyl	0.66 ± 0.10	0.46 ± 0.05**
32:0	1,2-Dipalmitoyl	6.62 ± 1.14	3.80 ± 0.65**
34:1–2	1-Palmitoyl-2-oleoyl, 1-palmitoyl-2-linoleoyl	14.68 ± 0.59	14.84 ± 1.43
34:0	Palmitoyl-stearoyl	5.62 ± 0.29	7.31 ± 1.10*
36:4	1-Palmitoyl-2-arachidonoyl	4.73 ± 0.18	2.86 ± 0.51***
36:2–3	1,2-Dioleoyl, oleoyl-linoleoyl	4.66 ± 0.33	6.40 ± 1.08**
36:1–2	1-Stearoyl-2-oleoyl, 1-stearoyl-2-linoleoyl	14.10 ± 0.44	20.94 ± 1.99***
38:3–4	1-Stearoyl-2-arachidonoyl	47.21 ± 2.09	42.73 ± 4.59*
40		1.70 ± 0.93	0.66 ± 0.85

Values are mean ± S.D. *, ** and *** denote significant ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively) differences between corresponding groups.

starvation (Table 2). These differences were of the same type as those seen in the submaxillary gland under the same condition, but they were more marked. However, the differences were smaller than the difference previously observed in pancreas after pair-feeding these diets for 24 days [2, 3]. This was also the case with the differences seen when three days of feeding control diet followed pair-feeding the test diets for 24 days.

Effects of secretagogues. Infusion of caerulein had no significant effects. Infusion of carbamyl-β-methylcholine also failed to cause any major changes although statistically significant changes were noted at least for the composition in submaxillary gland (Table 3). The abundance of the minor arachidonoyl-containing species (36:4 fraction) was increased whereas the percentage of the major arachidonoyl-containing species, constituting most of the 38:3–4 fraction, was not affected by the secretagogue. The treatment increased the 36:2–3 fraction in both organs.

Discussion

The results of the present study confirm the effect of chronic ethanol administration on the composition of the phosphatidylinositols in pancreas and submaxillary gland [2, 3]. The composition of phosphatidylinositols in liver [2] and testis (T. Cronholm and T. Curstedt, unpublished observations) were essentially unchanged after chronic administration of ethanol. Since chronic administration of ethanol causes decreased responses both in pancreas and salivary glands [12, 13], this indicates that the effect is related to the role of phosphatidylinositol in stimulus-secretion coupling [4–8].

The change might be due to the increased basal secretion that is seen in both glands after chronic ethanol administration [12, 13]. Increased turnover in a phosphoinositide cycle with a limiting rate in the acyl exchange reactions [14] would result in more similar acyl compositions in diacylglycerol and phosphatidylinositol. In order to test this hypothesis rats were given the secretagogues caerulein and carbamyl-β-methylcholine by i.v. infusion in doses known to stimulate secretion [10, 11]. Changes in acyl composition were observed in both organs after stimulation of muscarinic receptors, but these changes were not similar to those seen in ethanol-fed animals. Thus carbamyl-β-methylcholine caused an increase in the fraction of a minor arachidonoyl-containing species without affecting the abundance of the major arachidonoyl-containing species whereas the ethanol-fed rats had decreased percentages of both these species. On the other hand, the abundance of the species having 36 acyl carbons and 2 or 3 double bonds changed in the same direction by both treatments. It is concluded that the changes seen in the ethanol-fed rats were not due to increased basal secretion.

It is possible that the diacylglycerols and phosphatidic acids which are formed in the phosphoinositide cycles do not normally mix with the general pools of these compounds [5, 15]. It may be speculated that ethanol interferes with this compartmentation. This would cause dilution with the diacyglycerol backbone from other lipids and thus decrease the content of arachidonic acid in phosphatidylinositol.

The decrease in arachidonoyl-containing phosphoinositides may have several consequences. Since these molecular species appear to be specifically involved in the mediation of cellular responses [16, 17], the results could explain the decreased secretion response in pancreas and submaxillary gland after a given stimulus [12, 13]. The attenuation of the secretion response might also be due to decreased production of arachidonic acid, and thus of prostaglandin E_2, causing a smaller relaxant effect on the pancreatic ducts [18]. Mechanisms involving diacylglycerols, inositol phosphates and prostaglandins have probably different end-effects on the secretory process. Changes in fatty acid composition might change the balance between these different end-effects, ultimately causing pancreatic disease. This effect could be enhanced by the decreased production of prostaglandin E_2, since this compound protects against ethionine-induced pancreatitis [19, 20]. The cell damage might affect the compartmentation of the constituents of phosphoinositide cycles, and if interference with this compartmentation is caused by ethanol as suggested above, these effects might constitute a vicious circle that could result in pancreatitis.

In summary, the difference in abundance of arachidonoyl-containing phosphatidylinositols in pancreas and submaxillary gland between rats fed an ethanol-containing diet for 24 days and pair-fed controls persisted to some extent three days after shifting to control diet. One week of feeding the diets was sufficient for production of significant differences. Infusion of carbamyl-β-methylcholine increased the fraction of a minor arachidonoyl-containing species which was decreased in ethanol-fed animals. The results indicate that the changes observed after ethanol administration are not due to increased turnover in phosphoinositide cycles. It is speculated that the changes are due to interference with compartmentation of constituents of these cycles and that they might be of significance for the development of pancreatitis.

Table 2. Time-course of changes in composition of phosphatidylinositols in pancreas and submaxillary gland of ethanol-fed rats

Acyl carbons: double bonds	Percentage composition in pancreas: Liquid diet 6 days, starved 1 day, Control (N = 5)	Liquid diet 6 days, starved 1 day, Ethanol (N = 5)	Liquid diet 24 days, control diet 3 days, Control (N = 5)	Liquid diet 24 days, control diet 3 days, Ethanol (N = 5)	Percentage composition in submaxillary gland: Liquid diet 6 days, starved 1 day, Control (N = 5)	Liquid diet 6 days, starved 1 day, Ethanol (N = 5)	Liquid diet 24 days, control diet 3 days, Control (N = 5)	Liquid diet 24 days, control diet 3 days, Ethanol (N = 5)
32:1	0.11 ± 0.05	0.45 ± 0.34	0.18 ± 0.03	0.18 ± 0.05	0.45 ± 0.13	0.46 ± 0.12	0.50 ± 0.04	0.71 ± 0.40
32:0	2.76 ± 0.38	1.71 ± 0.21***	2.05 ± 0.25	1.72 ± 0.20*	7.30 ± 0.15	6.67 ± 1.22	5.33 ± 0.31	4.23 ± 0.51**
34:1–2	9.38 ± 1.03	9.85 ± 1.86	7.38 ± 0.73	7.80 ± 0.85	15.33 ± 0.64	14.62 ± 0.81	13.07 ± 0.70	13.02 ± 1.04
34:0	5.87 ± 1.07	6.94 ± 2.16	4.65 ± 0.45	5.24 ± 0.43	5.62 ± 0.49	7.07 ± 1.64	4.96 ± 0.65	6.61 ± 0.34**
36:4	2.20 ± 0.08	1.25 ± 0.30***	3.93 ± 0.53	3.41 ± 0.40	3.81 ± 0.34	2.75 ± 0.63*	4.36 ± 0.32	3.09 ± 0.26***
36:2–3	3.07 ± 0.36	2.77 ± 0.36	4.40 ± 0.52	5.38 ± 0.51*	3.50 ± 0.38	3.21 ± 0.60	4.89 ± 0.28	5.13 ± 0.31
36:1–2	28.14 ± 1.33	38.42 ± 2.78***	19.09 ± 2.90	23.21 ± 1.78*	14.50 ± 1.05	17.67 ± 0.91***	12.77 ± 1.20	17.25 ± 1.03***
38:3–4	46.76 ± 2.76	35.85 ± 3.57***	56.86 ± 3.82	48.21 ± 1.97**	47.93 ± 1.48	46.52 ± 2.73	53.41 ± 1.80	48.38 ± 2.23**
40	1.71 ± 0.98	2.75 ± 1.05	1.46 ± 0.73	4.85 ± 0.89***	1.56 ± 0.63	1.03 ± 1.16	0.73 ± 0.59	1.60 ± 1.18

Values are mean ± S.D. *, ** and *** denote significant ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively) differences between corresponding groups.

Table 3. Effects of intravenous infusion of carbamyl-β-methylcholine on the composition of phosphatidylinositols in pancreas and submaxillary gland of rats

Acyl carbons: double bonds	Percentage composition in pancreas: Infusion for 18 hr, Control (N = 4)	Infusion for 18 hr, Carbamyl-β-methylcholine (N = 4)	Infusion for 4 days, Control (N = 4)	Infusion for 4 days, Carbamyl-β-methylcholine (N = 4)	Percentage composition in submaxillary gland: Infusion for 18 hr, Control (N = 4)	Infusion for 18 hr, Carbamyl-β-methylcholine (N = 4)	Infusion for 4 days, Control (N = 4)	Infusion for 4 days, Carbamyl-β-methylcholine (N = 4)
32:1	0.29 ± 0.04	0.36 ± 0.08	0.52 ± 0.09	0.46 ± 0.02	0.36 ± 0.12	0.69 ± 0.06**	1.00 ± 0.12	0.96 ± 0.09
32:0	2.77 ± 0.27	3.18 ± 0.86	4.19 ± 0.76	3.98 ± 0.13	6.80 ± 0.13	6.80 ± 1.09	7.88 ± 1.14	7.11 ± 0.83
34:1–2	9.11 ± 0.95	11.42 ± 1.65	12.14 ± 1.35	13.21 ± 0.42	14.25 ± 1.08	16.29 ± 0.78**	14.73 ± 0.46	15.46 ± 1.39
34:0	5.25 ± 0.77	5.15 ± 0.87	7.09 ± 0.39	6.05 ± 0.22**	6.06 ± 0.75	5.68 ± 0.42	6.51 ± 0.81	5.49 ± 0.35
36:4	2.50 ± 0.10	3.13 ± 0.40*	3.73 ± 0.24	4.05 ± 0.14	3.04 ± 0.25	4.69 ± 0.27***	3.98 ± 0.21	5.18 ± 0.43**
36:2–3	3.81 ± 0.60	5.19 ± 0.36**	5.01 ± 0.33	5.54 ± 0.55	2.64 ± 0.29	4.37 ± 0.58**	4.81 ± 0.11	5.96 ± 0.52**
36:1–2	22.53 ± 1.01	21.44 ± 0.69	23.09 ± 0.76	23.12 ± 0.55	15.60 ± 0.90	12.27 ± 0.96**	13.05 ± 0.53	11.37 ± 0.97*
38:3–4	48.58 ± 2.65	46.88 ± 2.30	40.13 ± 2.54	40.09 ± 0.86	47.45 ± 2.21	47.52 ± 3.13	43.49 ± 2.37	45.35 ± 2.93
40	5.17 ± 1.27	3.27 ± 0.93	4.11 ± 0.14	3.51 ± 0.65	3.81 ± 0.53	1.72 ± 0.79**	4.56 ± 0.30	3.13 ± 0.47**

Values are mean ± S.D. *, ** and *** denote significant ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively) differences between corresponding groups.

Acknowledgements—The skilful technical assistance of Margareta Viestam-Rains is gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (No. 25X-2189), the Bank of Sweden Tercentenary Foundation and Karolinska Institutet.

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Biochemical Pharmacology, Vol. 35, No. 9, pp. 1611–1613, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

Reserpine inhibition of lipid peroxidation and protein phosphorylation in rat brain

(*Received* 28 *November* 1984; *accepted* 24 *October* 1985)

Reserpine has been used as a pharmacological agent and as a tool to study turnover of monoamines in CNS for a long time. However, its mode of action at a molecular level is still not properly understood. Reserpine presumably acts on the membranes of the intraneuronal vesicles by a process which is ATP and Mg^{2+} dependent [1].

A number of psychotropic drugs, especially the phenothiazines, affect lipid peroxidation [2] as well as protein phosphorylation in cerebral preparations [3]. Here we report that reserpine is a very strong inhibitor of lipid peroxidation in rat brain homogenates and mitochondrial preparations and that it also affects protein phosphorylation in cerebral cortex slices as measured by ^{32}P incorporation.

Lipid peroxidation in homogenate or mitochondrial preparations of brain was determined as described earlier [4] in the presence or absence of reserpine. For *in vivo* experiments, rats were given reserpine (CIBA, India), 2 mg/kg, intraperitoneally for 1 day (acute) or 5 days (chronic). The effect of reserpine on some $O_2^{\cdot}$ (superoxide radical) mediated reactions was studied in a system containing epinephrine (3 mM) and EDTA (1 mM) in sodium carbonate buffer (0.05 M) at pH 10.2 as described [5]; the initial rate of formation of adrenochrome was followed at 480 nm. In another set of experiments, formation of diformazan from phenazine methosulfate (PMS) in the presence of nitroblue tetrazolium (NBT) and NADH was measured in the absence or presence of reserpine (10 μM). The final assay mixture contained 16 mM Tris–HCl buffer, pH 8.0, 73 μM NADH, pH 8.0, in Tris buffer, 5.2 μM PMS in water, 80 μM NBT in water with or without reserpine (10 μM). The absorbancy was read at 560 nm at 10 min after the onset of reaction [6]. Protein phosphorylation was measured exactly as described by Rodnight *et al.* [7] in rat cerebral cortex slices prepared according to Shankar and Quastel [8], except that the period of incubation was 1 hr. ^{32}P (15 μCi) was present right from the beginning of incubation. Reserpine (50 or 100 μM), when present, was added at the start of incubation. Protein bound phosphorus was determined by the method of Martin and Doty [9]. Protein was estimated by the method of Lowry *et al.* [10].

Results given in Table 1 show that reserpine at concentrations of 25, 50 and 100 μM effectively inhibited *in vitro* lipid peroxidation of brain homogenate. With 100 μM reserpine, the *in vitro* peroxidation was inhibited almost completely. With 25 or 50 μM reserpine, the *in vitro* peroxidation was reduced by more than 30 and 50% respectively. The extent of peroxidation was determined after 90 min of incubation at 37° in the presence or absence of the drug.

The time course of inhibition of mitochondrial lipid peroxidation by reserpine at 50 and 100 μM concentrations is shown in Fig. 1. Lipid peroxidation was initiated by 10 μM Fe^{2+}, and amounts of malonaldehyde formed at 0, 20, 40 and 60 min were measured. The inhibition was observed as early as at 20 min of incubation and was persistent throughout the period of incubation.

Chronic reserpinisation inhibited subsequent *in vitro* lipid peroxidation of the brain homogenate to a variable extent (20–40% compared to peroxidation of control samples after 90 min of incubation) in different sets of experiments (data not presented). Acute reserpinisation, i.e. a single injection of reserpine, did not seem to inhibit *in vitro* lipid peroxidation of brain homogenate as measured by malonaldehyde estimation (data not presented).

The effects of reserpine on $O_2^{\cdot}$ mediated reactions are presented in Table 2. Values from individual experiments