ACETYLCHOLINE STIMULATION OF SELECTIVE INCREASES IN STEARIC AND ARACHIDONIC ACIDS IN PHOSPHATIDIC ACID IN MOUSE PANCREAS

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Summary. During the acetylcholine-stimulated loss of phosphatidylinositol and gain in the level of phosphatidic acid in mouse pancreas, there is a selective increase in stearic and arachidonic acids in phosphatidic acid. The amounts parallel the decrease in phosphatidylinositol, which contains predominantly these two fatty acids. Addition of atropine to stimulated tissue reverses the changes. There is a selective disappearance of the stearoyl, arachidonoyl phosphatidic acid, and phosphatidylinositol increases. The changes support the hypothesis that the 1-stearoyl, 2-arachidonoyl diglyceride backbone of phosphatidylinositol becomes phosphatidic acid during acetylcholine stimulation, and is transformed back to phosphatidylinositol on reversion to the unstimulated state.

ACh[3] produces a net breakdown of PI in mouse pancreas; this is accompanied by an increase in the level of PA in the tissue; the levels of the other phospholipids do not change (1,2). The aim of the present work was to determine whether the fatty acid composition of the newly formed PA was compatible with its being derived from the PI which disappears. PI from brain and from liver has been shown to have a different fatty acid composition from that of the major phospholipids; it consists largely of 1-stearoyl, 2-arachidonoyl-*sn*-glycero-3-phosphorylinositol (3,4). We established that the PI of mouse pancreas also contains predominantly stearic and arachidonic acids in approximately equal proportions. In contrast, PA in the unstimulated mouse pancreas was found to contain only a very small propor-

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[3]Abbreviations used are: ACh - acetylcholine; PI - phosphatidylinositol; PA - phosphatidic acid; 18:0 - stearic acid; 20:4 - arachidonic acid; TLC - thin layer chromatography.

tion of these two fatty acids. We reasoned that, if the diglyceride backbone of PI was being converted to PA during ACh-stimulation, then the newly formed PA should differ from the PA in the control, unstimulated tissue, in that it would have an increased content of stearic and arachidonic acids, in approximately equal proportions. This was found to be the case.

METHODS

Male mice from an inbred Swiss-Webster strain were used. The pancreas tissue was removed and incubated as described previously (1,2). Phospholipids were extracted and subjected to prior fractionation on small silicic acid columns as described elsewhere (2). The final lipid separations were carried out on silica gel-impregnated glass fiber sheets obtained from Gelman Instrument Company, Ann Arbor, Michigan. PA was separated from the appropriate column fraction by one-dimensional chromatography on ITLC Type-SA sheets, ascending, with diisobutylketone - acetic acid - water - benzene, (80:24:3:1.5, v/v/v/v). PI was separated from the appropriate column fraction by one-dimensional chromatography on ITLC Type-SA sheets which had been dipped in 1mM sodium carbonate solution (aqueous) and air-dried. All sheets were activated by heating at 110°C for 30 minutes. The developing solvent was chloroform - methanol - acetic acid - water, (25: 12.5:4:0.2, v/v/v/v). The lipids were chromatographed as bands, with standard compounds chromatographed on either side of the band. The strips containing the standards were cut off and the standards, located by iodine vapor, were used as guides for cutting out the sample bands. PI was also extracted (5) and isolated on 500μm silica gel H:Mg-acetate TLC plates, which were developed in chloroform - methanol - ammonia (65:25:5, v/v/v) (6). PI was visualized on the TLC plates by spraying with 0.01% 2',7'-dichlorofluorescein in methanol. All extractions and chromatographic solvents contained 0.1% butylated hydroxytoluene as antioxidant.

PI samples which were scraped from TLC plates, and PI and PA samples which were cut from the glass fiber sheets, were immediately subjected to transesterification using 0.25-0.5 ml. of 1.5N HCl in anhydrous methanol at 60°C for 30 min. in vials of 3 ml. capacity, which were equipped with Teflon-lined screw caps. Heneicosanoic acid (21:0), obtained from Nu-Chek-Prep, Inc., Elysian, MN, was added as an internal standard before the transesterification was carried out. The fatty acid methy esters were extracted into 0.5 ml. of high purity hexane after addition of 0.2-0.4 ml. of deionized water.

Fatty acid methyl esters were resolved on a 6 ft. x 0.3 cm. i.d. glass gas chromatographic column of 15% diethylene glycol succinate on 80/100 mesh chromosorb W (Applied Science Laboratories, State College, PA), using hydrogen flame ionization detection. Fatty acids were identified by relative chain length values (7), using both saturated standards and fatty acid methyl esters derived from rat brain and liver total lipid extracts.

RESULTS

The predominant fatty acids of PI in mouse pancreas were found to be stearic and arachidonic acids; these two fatty acids were present in approximately equal proportions and they accounted for about 80% of the

total fatty acids in PI (Table 1). This composition is similar to that reported for PI in liver (3) and brain (4). The fatty acid composition of pancreas PI did not change significantly in tissue incubated either with or without ACh.

In control, unstimulated pancreas tissue, the PA had a net fatty acid composition which was very different from that of PI. Over 80% of the PA fatty acids were found to be oleic, linoleic and palmitic acids (Table 1). When PA formation occurred during ACh-stimulation, the main effect on fatty acid composition was a significant increase in moles per cent of stearic and arachidonic acids in PA (Table 1). Calculations of the levels of fatty acids in PA showed that the amounts of stearic and arachidonic acids increased by approximately 400% and 600%, respectively;

Table 1

Fatty acid composition of pancreas PI and PA from control and ACh-stimulated tissue

Fatty acid	*Moles per cent composition* PI[a]	PA[b] Control	PA[b] +10μM ACh	*P<*
Palmitic	6.7 ±0.3	18.4 ±2.9	21.1 ±2.6	
Palmitoleic	trace	6.8 ±0.9	4.9 ±0.7	
Stearic	41.2 ±1.1	4.1 ±1.1	14.3 ±1.5	*0.01*
Oleic	8.8 ±0.6	27.8 ±4.4	21.5 ±1.9	
Linoleic	5.1 ±0.5	37.3 ±5.4	25.3 ±4.8	
Arachidonic	36.7 ±1.5	2.0 ±0.4	9.5 ±0.4	*0.001*
Docosahexaenoic	trace	1.0 ±0.6	1.0 ±0.5	
Other	1.5 ±0.5	2.7 ±0.6	2.3 ±0.9	

[a]The fatty acid composition of the PI did not change significantly on incubation of the tissue either without or with ACh. Values for PI are the means ±SE from 8 individual pancreases.

[b]PA was isolated from tissues which had been incubated in Krebs-Henseleit glucose-bicarbonate medium, with 95% O_2 + 5% CO_2 as the gas phase at 38°C for 60 min without and with 10μM ACh (plus 0.1mM eserine). Three mouse pancreases were pooled for each incubation vessel. Values for PA are the means ±SE from 3 separate incubations and estimations. *P* values were derived from Student's *t* test (two-tailed); they express the significance of the difference between PA fatty acid values in unstimulated and stimulated pancreas.

there was a smaller percentage increase in palmitic acid (61%); the other fatty acids showed little or no change in levels (Table 2). The net increases in the levels of stearic, arachidonic and palmitic acids accounted for the net increase in the total PA level (Table 2). Because of the relatively high level of palmitic acid in the control PA, the absolute value for the increase in levels for this fatty acid is less reliable than the values for the increases in the levels of stearic and arachidonic acids. The main increases in fatty acid levels in PA in the ACh-stimulated tissue are compatible with a stearoyl-arachidonoyl-glycero-phosphoric acid structure for most of the newly-formed PA.

At concentrations of 10μM ACh or higher, and after 30 minutes or more of incubation, the increase in the level of PA in the tissue was shown previously to be, within experimental error, equal to the decrease in the level of PI (1,2). A comparison of the changes in the amounts of stearic and arachidonic acids in PI and PA after incubation with 100μM ACh for 80 min. showed that the loss of stearic and arachidonic acids from the PI fraction was accompanied by an almost stoichiometric gain in these two

Table 2

Levels of different fatty acids in PA after incubation without and with ACh

Fatty acid in PA	*nmoles/g. fresh tissue*		*Δ nmoles/g. tissue*	*% Difference*
	Control	+10μM ACh		
Palmitic	146 ± 3	235 ±26	+ 89	+ 61%
Palmitoleic	51 ± 9	53 ± 8	n.s.	n.s.
Stearic	31 ±10	162 ±12	+131	+422%
Oleic	212 ±47	240 ±14	n.s.	n.s.
Linoleic	222 ±12	201 ±25	n.s.	n.s.
Arachidonic	14 ± 3	100 ± 6	+ 86	+614%
Other	16 ± 2	21 ± 1	+ 5	+ 31%
Total PA fatty acids	694 ±24	1014 ±92	+320	+ 46%

Mouse pancreas tissue was incubated as in Table 1. Values are the means and ranges of 2 separate incubations and estimations for each treatment. n.s. - no significant difference.

fatty acids in the PA fraction (Table 3). The gains of stearic and arachidonic acid in PA were 72% and 89%, respectively, of the losses of these fatty acids from PI.

Previously it was also shown that, after incubation of mouse pancreas tissue with 10μM or 100μM ACh for 40 min., during which time the breakdown of PI and the appearance of new PA occurred, the addition of 1μM or 10μM atropine then caused the tissue to revert to the unstimulated

Table 3

Comparison of changes in stearic and arachidonic acid levels in PI and PA after ACh stimulation

Additions	*nmoles/g. fresh tissue* PI 18:0	PI 20:4	PA 18:0	PA 20:4
None	636	469	53	108
100μM ACh (+ 100μM eserine)	440	295	195	262
Δ due to ACh	-196	-174	+142	+154

Mouse pancreas tissue was incubated as in Table 1 for 80 min. Fatty acid estimations were carried out on PI and PA isolated from 3 pooled pancreases for each variable.

Table 4

Loss of stearic and arachidonic acids in PA on reversion to the unstimulated state

Incubation conditions	*nmoles/g. fresh tissue* Phosphatidic acid 18:0	20:4
100μM ACh, 0 min to 40 min	173 ±23	248 ± 9
100μM ACh, 0 min to 40 min, then 10μM atropine added at 40 min to 80 min	58 ±10	107 ±48
Δ due to atropine after ACh	-115	-147

Mouse pancreas tissue was incubated as in Table 1 for the time periods indicated. Values are the means ±SE of results from 3 incubations and assays. No other fatty acids in PA showed significant changes.

state, as manifested by a fall in the level of PA to that of the control, accompanied by a rise in the level of PI to that of the control (2). To test whether the PA which was lost under these conditions was acting as a precursor for PI synthesis, the changes in PA fatty acid composition after the addition of atropine to ACh-stimulated tissue were measured. The loss of PA during reversion to the unstimulated state involved the net loss of stearic and arachidonic acids in approximately equal amounts (Table 4). This net loss of stearic and arachidonic acids was similar in amount to the net increases in these two acids which occurred when the tissue was stimulated with ACh (Tables 2 and 3). No other fatty acids in PA showed a significant net loss during reversion to the unstimulated state.

These results are compatible with the hypothesis that a molecular species of PA which has a stearoyl, arachidonoyl composition is derived from PI breakdown during ACh stimulation and is used for the resynthesis of PI during reversion to the unstimulated state.

DISCUSSION

On the basis of isotope studies of PI metabolism in the transition to the ACh-stimulated state and the reversion to the unstimulated state in the avian salt gland, a cyclic interconversion of PI and PA was proposed in which the diglyceride moiety remained the same throughout (8-11). Because of the continuous turnover of the phosphate of PA in the stimulated state, without concomitant turnover of the glycerol moiety (12), the diglyceride itself was also assumed to be an intermediate in these cyclic interconversions. The unique fatty acid composition of PI has allowed us to test this hypothesis further in the pancreas, as reported here. The results support the hypothesis. They are compatible with a transfer of a 1-stearoyl, 2-arachidonoyl glyceride backbone of PI to the PA which is newly formed as a result of ACh-stimulation in the pancreas. They are also compatible with a transfer of a 1-stearoyl, 2-arachidonoyl PA back to PI on reversion of the tissue to the unstimulated state. The possible

role of 1-stearoyl, 2-arachidonoyl diglyceride as an intermediate in these interconversions, and the precise enzyme mechanisms by which these interconversions occur, remain to be determined.

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