Effect of Pilocarpine, Carbamylcholine and Pancreozymin on the Incorporation of ^{32}P into Phospholipids in Rat Pancreas Tissue in Vitro

by

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1. Introduction

The secretion of pancreatic juice is mainly regulated by hormonal and neurogenic factors [1, 2, 3, 4].

According to Becker, carbonic anhydrase, which is necessary to synthesize biocarbonate abundantly contained in pancreatic juice, seems to be closely related to the secretion of pancreatic juice in the mechanism of its flow [5].

Hokin et al. reported that after the injection of pilocarpine, the secretion of pancreatic juice was increased, while the synthesis of amylase was decreased [6]. Daly et al. made it clear that the digestive enzyme in pancreas decreased after the injection of pilocarpine [7].

Secretion will need energy as is generally said. This is supported by the fact that secretion is obstructed when there is no enzyme [8], that the uptake of O_2 rises when secretion is quickened by secretin and others [9], and that secretion is obstructed by presence of CN [10]. Despite the above facts, the secretion mechanism of pancreatic juice is not clear.

The author has examined what effects cholinergic agent (pilocarpine and carbamylcholine) and pancreozymin, which excites the pancreatic secretion of enzymes without any significant influence on the flow of water or bicarbonate [11], have on the phospholipids in rat pancreas tissue in vitro.

2. Material and Method

Animals; In this experiment, wister strain male rats, 6 weeks old, were used after being fasted for 24 hours.

Radioactive material; Solusion of $5\text{mCi H}_3^{32}\text{PO}_4$ in 1ml 0.01 N HcL was obtained from Daiichi Pure Chemicals Co. LTD., Tokyo.

Agents; Pilocarpine hydrochloride and carbamylcholine chloride were obtained from Daiichi Pure Chemicals Co. LTD., Tokyo.

Pancreozymin was obtained from Boots Pure Drug Co. LTD., England.

Pure phosphatidyl choline and lyso-phosphatidylcholine were obtained from Depart-

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Silica gel; Silica gel HR was obtained from E. Merck Ag, Darmstadt, W. Germany.

Reagents; 2, 5 diphenyloxazol (PPO) and dimethyl POPOP were obtained from Nakarai Chemicals LTD., Kyoto.

All other reagents used in this experiment were guaranteed reagents.

Medium; The medium used for the incubation of minced rat pancreas tissue was solution of Krebs & Henseleit.

Preparation and incubation of rat pancreas tissue:

Rat pancreas tissue was carefully removed from connective tissues and minced into approximately 2mm² before use. Immediately after mincing this tissue was placed in a covered crystallizing dish lying in chipped ice.

When minced tissue had sufficiently been prepared, they were weighed on a torsion balance and 1g of minced pancreas tissue was placed in flasks (each 50ml volume) each of which contained 10ml of medium. Pilocarpine or carbamylcholine was added to the medium to give a concentration of 0.1mg/10ml.

Pancreozymin was added to the medium to give a concentration of 1U/10 ml. ³²P was added to the medium to give a concentration of 10 μ Ci/10ml. The tissue was incubated for 2 hours at 37°C in an incubator (Taiyo Incubator K-II, Tokyo).

Immediately after incubation the tissues were removed from flasks and dried on filter paper (Tokyo Filter Paper No. 2)

The lipids were extracted by homogenizing the tissues in a waring blender containing chloroform-methanol mixture (2:1, V/V) according to the method of Folch, J. et al. [12].

Table 1. Isolation of the total lipids from rat pancreas tissue
(By method of Folch, J. et al.)

10ml Krebs & Henseleit solution containing 1g minced rat pancreas, 10 μ Ci³²P and pilocarpine, carbamylcholine or pancreozymin.

Method of assay:

The extracted total tissue lipids were dissolved in 0.5ml of anhydrous chloroform and separation of phospholipids from the total tissue lipids was carried out by T.L.C. (silica gel thin layer chromatography), the solvent being chloroform-methanol-water

Fig. 1. Chromatogram of the total lipids in the rat pancreas tissue.

(65:25:4, V/V). The silica gel layer of the phospholipids spots (phosphatidylethanolamine (PE), phosphatidyl choline (PC), sphingomyelin (Sph) and lyso-phosphatidyl choline (Lyso-PC) shown in Fig. 1) was cut out in 4 parts and extracted with 5ml of methanol respectively and its radioactivity was measured with liquid scintillation counter (Aloka Liquid Scintillation Counter, LSC-601, Tokyo).

Toluene-scintillator (PPO (2.50g) and dimethyl POPOP (0.150g) dissolved in toluene (500ml) was used in liquid scintillation.

The determination of phosphorus was carried out according to the method of Chen, P.S. et al. [13].

Spectrophotometer used for the determination of phosphorus was Spectrophotometer Model LS-7, Erma Optical Works, Tokyo.

3. Results

For ease of presentation, the results are to be given by means of tabulations below.

Tab. 2 shows that ^{32}P was incorporated into phospholipids in rat pancreas tissue in vitro, when 1g of minced rat pancreas tissue was incubated for 2 hours at 37°C in 10ml medium containing $10\mu\text{Ci}$ of ^{32}P .

Tab. 3 shows the effect of pilocarpine on the incorporation of ^{32}P into phospholipids.

In the case of pilocarpine, incorporation of ^{32}P into PE increased, that into Lyso-PC slightly increased in comparison with the control.

Tab. 4 shows the effect of carbamylcholine on the incorporation of ^{32}P into phospholipids.

In the case of carbamylcholine, incorporation of ^{32}P into PE increased, that into PC increased, that into Sph increased and that into Lyso-PC significantly increased in comparison with the control.

Tab. 5 shows the effect of pancreozymin on the incorporation of ^{32}P into phospholipids.

In the case of pancreozymin, incorporation of ^{32}P into PE, decreased, that into PC slightly increased, that into Sph decreased and that into Lyso-PC decreased in comparison with the control.

Table 2. Incorporation of ^{32}P into phospholipids in the rat pancreas tissue

		Control				mean $\pm$ SE
PE	P_{γ}	0.56	0.73	0.60	0.48	0.59 ± 0.05
	cpm.	62	71	62	49	61.00 ± 4.52
	Spec.	110.71	97.26	103.33	102.08	103.35 ± 2.78
PC	P_{γ}	2.71	3.24	3.02	2.70	2.92 ± 0.13
	cpm	54	97	66	54	67.75 ± 10.15
	Spec.	19.93	29.94	21.85	20.00	22.93 ± 2.37
Sph	P_{γ}	0.70	0.56	0.61	0.72	0.65 ± 0.04
	cpm	62	50	53	78	60.75 ± 6.28
	Spec.	88.57	89.29	86.89	108.33	93.27 ± 5.04
Lyso-PC	P_{γ}	1.62	1.15	1.01	1.03	1.20 ± 0.14
	cpm	106	69	70	73	79.50 ± 8.87
	Spec.	65.43	60.00	69.31	70.87	66.40 ± 2.42
Tp	P_{γ}	20.75	19.17	18.57	16.80	18.82 ± 0.82
	Spec.	117	115	114	113	114.75 ± 1.48
		5.64	6.00	6.14	6.73	6.13 ± 0.23

Phosphatidylethanolamine Lyso-PC; Lyso-phosphatidylcholine
PC ; Phosphatidylcholine Tp ; Total lipids
Sph; Sphingomyelin Spec ; Specific radioactivity

Table 3. Effect of pilocarpine on the incorporation of ^{32}P into phospholipids in the rat pancreas tissue

		Pilocarpine 0.1mg/10ml medium				mean $\pm$ SE
PE	P_{γ}	0.63	0.60	0.92	0.82	0.74 ± 0.08
	cpm	77	71	114	87	87.25 ± 9.51
	Spec.	122.22	118.33	123.91	106.10	117.64 ± 4.02
PC	P_{γ}	2.87	2.07	3.75	3.68	3.08 ± 0.40
	cpm	68	45	77	73	65.75 ± 7.15
	Spec.	23.69	21.74	20.52	19.84	21.45 ± 1.46
Sph	P_{γ}	0.39	0.22	1.04	0.95	0.44 ± 0.09
	cpm	50	42	78	58	57.00 ± 9.46
	Spec.	128.21	190.91	116.42	138.10	143.41 ± 16.44
Lyso-PC	P_{γ}	0.92	0.63	1.04	0.95	0.89 ± 0.09
	cpm	53	46	86	72	64.25 ± 0.09
	Spec.	57.61	73.02	82.69	75.79	72.28 ± 5.29
Tp	P_{γ}	21.9	20.7	32.3	24.7	24.88 ± 2.60
	cpm	68	72	76	72	72.00 ± 1.63
		3.11	3.48	2.35	2.91	2.96 ± 0.47

Table 4. Effect of carbamylcholine on the incorporation of ^{32}P into phospholipids in the rat pancreas tissue

		Carbamylcholine 0.1mg/10ml medium				mean $\pm$ SE
PE	$\text{P}\gamma$	0.36	1.04	0.76	0.75	0.73 ± 0.13
	cpm	61	146	117	81	101.25 ± 18.88
	Spec.	96.83	140.38	153.95	108.00	124.79 ± 13.40
PC	$\text{P}\gamma$	2.47	4.60	3.14	2.88	3.14 ± 0.34
	cpm	77	117	113	110	104.25 ± 9.19
	Spec.	31.17	25.43	35.99	38.19	32.70 ± 2.45
Sph	$\text{P}\gamma$	0.44	1.01	0.69	0.50	0.66 ± 0.13
	cpm	65	115	82	74	84.00 ± 10.90
	Spec.	147.73	113.86	118.84	148.00	132.10 ± 9.15
Lyso-PC	$\text{P}\gamma$	0.48	1.14	1.01	0.83	0.93 ± 0.14
	cpm	78	117	115	98	102.00 ± 9.06
	Spec.	162.50	102.63	113.86	118.07	124.27 ± 13.15
Tp	$\text{P}\gamma$	16.1	24.3	22.2	18.8	20.35 ± 1.81
	cpm	84	117	117	109	106.75 ± 7.81
	Spec.	5.22	4.81	5.27	5.80	5.28 ± 0.14

Table 5 Effect of pancreozymin on the incorporation of ^{32}P into phospholipids in the rat pancreas tissue

		Pancreozymin 1U/10ml medium				mean $\pm$ SE
PE	$\text{P}\gamma$	0.93	0.87	0.42	0.48	0.68 ± 0.13
	cpm	66	52	41	56	53.75 ± 5.17
	Spec.	66.83	59.19	97.85	115.62	84.87 ± 13.22
PC	$\text{P}\gamma$	1.70	2.55	1.20	2.30	1.94 ± 0.30
	cpm	60	56	45	59	55.00 ± 3.43
	Spec.	35.35	22.11	37.08	25.47	30.00 ± 3.66
Sph	$\text{P}\gamma$	2.00	2.43	2.10	1.65	2.05 ± 0.11
	cpm	55	53	32	46	46.50 ± 5.20
	Spec.	27.40	21.89	15.14	29.67	23.53 ± 3.23
Lyso-PC	$\text{P}\gamma$	1.45	1.77	2.07	1.85	1.81 ± 0.13
	cpm	50	96	52	52	62.50 ± 11.17
	Spec.	34.55	54.35	25.26	27.89	35.51 ± 5.69
Tp	$\text{P}\gamma$	23.8	6.5	10.9	15.4	14.15 ± 3.42
	cpm	70	74	92	118	88.50 ± 10.93
	Spec.	2.93	11.41	8.47	7.63	7.61 ± 1.75

4. Discussion

As the result of the author's experiment shown in Fig. 2, it is found that in the patterns of effects of incorporation of ^{32}P into phospholipids there is a great difference between the two groups,—juice flow-accelerating agents (pilocarpine and carbamylcholine) and pancreatic enzyme secretion-accelerating hormone (pancreozymin which has been known to have no significant influence on the flow of water or bicarbonate).

In the case of the latter, each incorporation of ^{32}P into phospholipids decreases in comparison with the control.

In the former two agents, especially in the case of carbamylcholine, it is particularly noted that the incorporation of ^{32}P into lysophosphatidylcholine significantly

Fig. 2. Total effects of incorporation of ^{32}P into the phospholipids

increases, but that the incorporation of ^{32}P into phosphatidylcholine slightly increases.

Therefore, this phenomenon is considered to indicate that lysophosphatidylcholine is synthesized *de novo* by the direct or indirect action of carbamylcholine, on the one hand, and that lyso-phosphatidylcholine is produced when phospholipase A acts on phosphatidylcholine, on the other hand.

Accordingly phospholipase A seems to take part in the above change. And it is observed that this phospholipase A spreads fairly widely in the mammal tissues, for example, and that there is a high degree of its activity in the pancreas and small intestine, some degree of it in the testicle, spleen, lung and liver, and a small degree of it in the brain, heart-muscle and skeletal-muscle [14, 15].

And Magee et al. have found phospholipase A in both the fresh and frozen-stored tissues of human pancreas [16].

Several effects after injection of phospholipase A into animals have been reported, e.g. a decrease in plasma phosphatidylcholine, an increase in plasma lyso-phosphatidylcholine, capillary permeability enhanced, a decrease in the phosphatidylcholine content of muscle at the site of injection and significant myolysis [17, 18, 19].

Higuchi has found that substances which activate the phospholipase A, namely anionic amphipathic substance (dicetyl phosphate, dodecyl sulfate, hexadecyl sulfate and taurocholate) increase the secretion of pancreatic juice [20, 21].

Osada has reported that anionic amphipathic substance increases not only the bilious secretion, but parotid salivary secretion [22].

Yazawa has recognized that substances which inhibit the phospholipase A activity, EDTA, cysteine, lead acetate and glutathion decrease the bilious secretion [23, 24].

Carbamylcholine increases the secretion of pancreatic juice, and so when this fact is considered, together with the effect of carbamylcholine on incorporation of ^{32}P into phospholipids, it may be said as the result of the author's experiment, that phospholipase A takes part in the secretion of pancreatic juice.

But in the case of pilocarpine, the incorporation of ^{32}P into lysophosphatidylcholine and phosphatidylcholine is almost similar to that of the control, so it is doubtful that phospholipase A may have a role in the pancreatic juice secretion process.

The pattern of incorporation of ^{32}P into phosphatidylethanolamine and sphingomyelin are quite similar between the pilocarpine and carbamyl choline groups, showing that the mechanism of both effects may probably be the same.

The mechanism of both effects, above noted, can not be clarified by the present experiment, but it has been reported that the O_2 uptake rises during the quickened secretion [9], and among the cellular respiratory enzymes there are some enzymes with phospholipids in their molecular, such as cytochrome oxydase [25, 26, 27].

The oxidative processes take place mainly in the mitochondria which are closely connected with phospholipids.

Considering these facts, it is not yet clear that the elevated incorporation of ^{32}P into phosphatidylethanolamine and sphingomyelin in both cases of pilocarpine and carbamylcholine groups may be due to some indirect effects concerning phospholipids, so the author thinks that this present study may give a fundamental significance to the phospholipid physiology in the field of pancreatic secretion.

5. Summary

1. In the method described in the text, incorporation of ^{32}P into phospholipids (PE, PC, Sph and Lyso-PC) in rat pancreas tissue was observed.

2. In the case of pilocarpine, the incorporation of ^{32}P into each phospholipid showed a tendency of increasing in comparison with the control.

3. In the case of carbamylcholine, the incorporation of ^{32}P into phospholipids generally increased, especially that into Lyso-PC remarkably in comparison with the control.

4. In the case of pancreozymin, the incorporation of ^{32}P into phospholipid showed generally a tendency of decreasing in comparison with the control except that into PC.

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References

- [1] DELANEY, J. P. and GRIN, E.; Influence of hormones and drugs on canine pancreatic blood flow *Amer. J. Physiol.* 211, 1398–1402, (1966).
- [2] HENRIKSEN, F. W.; Effect of Pancreozymin on the canine pancreatic secretion of fluid and bicarbonate. *Scand. J. Gastroent.* 3, 637–640, (1968).
- [3] SUDA, Y., ROBINSON, L. A. and WHITE, T. T.; The effects of adrenergic blocking agents on pancreatic secretion in dogs. *Annals of Surgery*, April, 625–630, (1969).
- [4] TANKEL, H. and HOLLANDER, F.; The relation between pancreatic secretion and local blood flow. A Review: *Gastroenterology*, 32, 633–641, (1957).

- [5] BECKER, V.: The exocrine pancreas. Ciba Foundation Symposium. Little Brown & Co. Boston, 1961
- [6] HOKIN, L. E.; The synthesis and secretion of amylase by pancreas in vitro. *Biochem. J.* 48, 320–326, (1943).
- [7] DALY, M. M. and MIRSKY, A. E.; Foundation of protein in the pancreas. *J. Gen. Physiol.* 36, 243–254, (1952).
- [8] CHADON, G. and Gross, A.; Influence de l'insuline sur la secretion pancreatique externe. *Compt. Rend. Soc. Biol.* 140, 1004, (1946).
- [9] DAVISE, R. E., HARPER, A. A. and MACKAY, I. F. S.; A comparison of the respiratory activity and histological changes in isolated pancreatic tissue. *Amer. J. Physiol.* 157, 278–282, (1949).
- [10] HOKIN, L. E.; Amino acid requirements of amylase synthesis by pigeon pancreas slices. *Biochem. J.* 50, 216–220, (1951).
- [11] HARPER, A. A. and PAPER, H. S.; Pancreozymin, a stimulant of the secretion of pancreatic enzymes in extracts of the small intestine. *J. Physiol.* 102, 115–125, (1943).
- [12] FOLCH, J., LEES, M. and SLOANESTANLY, G. H.; A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497–509 (1957).
- [13] CHEN, P. S. JR., TORIBARA, T. Y. and WARNER, H.; Microdetermination of phosphorus. *Anal. Chem.* 28, 1756–1758, (1956).
- [14] ROBERTSON, A. F. and LANDS, W. E. M.; Positional specificities in phospholipid hydrolyses. *Biochemistry*, 1, 804–810 (1962).
- [15] GALLAI-HACHARD, J. J. and THOMSON, R. H. S.; Phospholipase A activity of mammalian tissues. *Biochim. Biophys. Acta*, 98, 128–136, (1965).
- [16] MAGEE, W. L., GALLAI-HACHARD, J. J., SANDERS, H. and THOMPSON, R. H. S.; The purification and properties of phospholipase A from human pancreas. *Biochem. J.* 83, 17–25, (1962).
- [17] MAENO, H., MITSUHASHI, S., OKONOJI, T., HOSHI, S. and HONMA, M.; Studies on Habu snake Venom V myolysis caused by phospholipase A in Habu snake venom. *Jap. J. Exp. Med.* 32, 55–64, (1962).
- [18] KLIBANSKY, C., CONDREA, E. and DEVRIES, A.; Changes in plasma phospholipids following intravenous phosphatidase A injection in the rabbit. *Bull. Res. Coun. Israel.* IOE Nos 3–4 (1963).
- [19] ROSENBERG, D. and PODLESKI, T. R.; Block of conduction by acetylcholine and d-Tubocurarine after treatment of Squid Axon with cotton-mouth moccasin venom. *J. Pharmac. Exp. Ther.* 137, 249–262, (1962).
- [20] HIGUCHI, M.; The influence of anionic amphipathic substances on the secretion of pancreatic juice in rabbit. (Unpublished.)
- [21] DAWSON, R. M. C.; The influence of the physical nature of substrate on phospholipase activity. *Collog. Ges. Physiol. Chem.* 16, 29–39, 1968
- [22] OSADA, H.; Studies on substances influencing the secretion of parotid saliva and hepatic bile in guinea pig. *J. Nihon Univ. Dent. J.* in press
- [23] YAZAWA, H.; The influence of substances inhibiting the phospholipase A activity on the bilious secretion in guinea pig. (Unpublished)
- [24] MARINETTI, G. V.; The action of phospholipase A on lipoproteins. *Biochem. biophys. acta*, 98, 554–565, (1965).
- [25] MARINETTI, G. V.; Scaramuzzino, D. J. and Stotz, E.; Lipides of cytochrome oxydase. *J. Biol. Chem.* 224, 819–826, (1957).
- [26] MARINETTI, G. V., KOCHEN, J. ERBLAND, J. and STOTZ, E.; The lipide composition of purified cytochrome preparation of pig heart. *J. Biol. Chem.* 229, 1027–1035, (1957).
- [27] MARINETTI, G. V., ERBLAND, J., KOCHEN, J. and STOTZ, E.; The phosphatide composition of a purified cytochrome oxydase preparation. *J. Biol. Chem.* 233, 740–742, (1958).