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EFFECTS OF ACETYLCHOLINE ON THE INCORPORATION OF P^{32} INTO THE PHOSPHOLIPIDS IN SLICES OF SKIN FROM CHILDREN WITH AND WITHOUT CYSTIC FIBROSIS OF THE PANCREAS *

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Stimulation of the secretion of a variety of water-soluble substances in endocrine and exocrine glands by appropriate secretory stimulants is associated with a characteristic increase in the metabolism of certain phosphatides, particularly phosphoinositide (phosphatidyl inositol) and phosphatidic acid [see review (1)]. Among the tissues where this relationship between secretion and phospholipid metabolism has been observed is the avian salt gland, which secretes hypertonic solutions of sodium chloride in response to acetylcholine (2). This suggested that a similar relationship might exist between phospholipid metabolism and sweat secretion in sweat glands.

The secretion of sweat by the sweat glands of human skin is under cholinergic control. Normal human sweat is hypotonic. In healthy children, sweat is markedly hypotonic, but in those suffering from cystic fibrosis of the pancreas, the concentrations of sodium and of chloride approach isotonicity (3). Since many of those tissues showing a phospholipid effect are also those involved in cystic fibrosis of the pancreas (e.g., the pancreas and salivary glands), investigations were undertaken to determine whether a phospholipid effect could be evoked in skin from control patients and in patients with cystic fibrosis and whether this effect might differ in the two groups.

In the present investigation, the rate of sweating and the sodium concentration of the sweat were measured in control children and in children with cystic fibrosis of the pancreas after iontophoresis of pilocarpine into the forearm. Biopsies of skin were made, and the incorporation of P^{32} into the phosphatides was measured in slices of skin incubated without and with acetylcholine (with eserine). It was found that in slices of skin acetylcholine approximately doubled the incorporation of P^{32} into phosphatidic acid and phosphoinositide after a 2-hour incubation period. There were slight increases in the incorporation of P^{32} into phosphatidyl choline and phosphatidyl ethanolamine. The incubation of skin with acetylcholine is thus associated with a "phospholipid effect" that is probably associated with stimulation of sweat gland activity. This is qualitatively very similar to what has been observed in other glandular tissues.

The phospholipid effect in skin from children with cystic fibrosis of the pancreas did not differ significantly from that in skin from control children.

One undernourished child of short stature who was initially anhidrotic and could not be stimulated to sweat with pilocarpine was also studied. This was the only child out of 15 investigated in whom we observed no increase in the incorporation of P^{32} into the phosphatides on incubation of slices of skin with acetylcholine. This provides evidence that the phospholipid effect in skin is in fact associated with the secretion of sweat.

METHODS

Diagnosis of cystic fibrosis of the pancreas. All the children in this series were studied clinically by one of us (CCL). The children diagnosed as having cystic fibrosis of the pancreas had the pulmonary and gastrointestinal symptoms characteristic of the disease. Sweat tests and estimations of sodium in the sweat were performed as described previously (4). The mean rate of sweat secretion in control children was 4.72 ± 1.00 g per m^2 per minute, and in children with cystic fibrosis of

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the pancreas it was 7.14 ± 0.49 g per m^2 per minute. These differences were not significantly different. In a larger series (4), the means in the two groups showed closer agreement.

Preparation and incubation of skin tissue. An oval piece of skin about 5×10 mm extending down to the subcutaneous tissue was removed under aseptic conditions (with xylocaine anesthesia) from an area midway between the xiphoid and the umbilicus just lateral to the mid-line. The tissue was immediately placed in a chilled crystallizing dish and transported from the operating room to the laboratory. The subcutaneous fat was removed from the under surface of the skin, and slices were prepared with the microtome of Stadie and Riggs (5). Three 0.5-mm slices were generally made from a piece of skin. Each of these was divided into approximately six equal portions. Single portions from each of the three slices were pooled in six batches. Thus, each of the six vessels contained an equal amount of tissue derived from each of the three layers of skin.

The tissues were incubated in 25-ml Erlenmeyer flasks containing 1.0 ml of bicarbonate saline (6) treated with 5% CO_2 and 95% O_2 and containing 120 mg per 100 ml glucose and aproximately 60 μc of P^{32}. Acetylcholine (10^{-5} M) and eserine (10^{-4} M) were added to the even-numbered vessels. Thus, for a given biopsy, triplicate incubations without and with acetylcholine were performed. The tissues were incubated for 2 hours at 37° C.

When the incorporation of inositol-2-H^3 into lipids was studied, the slices were preincubated for 15 minutes for three successive periods in 1.0 ml of bicarbonate saline containing 120 mg per 100 ml glucose to remove as much endogenous inositol as possible. They were then incubated as above, except that the vessels contained 40 mg per 100 ml of inositol-2-H^3 (SA, 300 μc per μmole) instead of P^{32}.

Determination of radioactivity in the phospholipids. The tissues incubated with P^{32} were treated as follows. After incubation the tissues were placed in 50-ml test tubes and frozen. A 4.0-ml volume of 1:1 chloroform-ethanol was then added, and the mixture was refluxed at 70° C for 2 hours with a "cold-finger" condenser. After extraction of the phospholipids, the chloroform-ethanol extracts (without tissue) were poured into conical centrifuge tubes and washed twice with 5 ml of cold 0.1 N HCl as described previously (7). Samples of the chloroform phase were chromatographed either by the method of Marinetti, Erbland, and Kochen (8), or by the method of Beiss and Armbruster (9). Radioautograms were then made, the radioactive phosphatide spots were cut out, and they were counted as described previously (7). The radioactivities of the phosphatides are expressed as counts per minute per 100 mg of fresh tissue, corrected to 10^6 cpm per μg of phosphorus for the inorganic phosphorus in the medium.

Tissues incubated with inositol-2-H^3 were treated as follows. The slices were ground with sand and made up to 5 ml with water. A 0.5-ml volume of 50% trichloroacetic acid was added, and the suspension was centrifuged and washed once with 5% trichloroacetic acid. A 4.0-ml volume of 1:1 chloroform-ethanol was then added, and the lipids were allowed to extract overnight in tightly stoppered tubes at 2° C. The next day, the extracts were washed with 0.1 N HCl as above. Samples of the chloroform phase were dried in scintillation vials under nitrogen and then *in vacuo* over KOH. Ten ml of toluene containing 0.4% 2,5-diphenyloxazole and 0.005% 1,4-bis-2(5-phenyloxazolyl)-benzene were then added. Radioactivities in the unknown samples, in a tritium-labeled toluene standard, and in a blank sample were determined in a Packard Tri-Carb scintillation spectrometer; they were then corrected to give disintegrations per minute by the addition of a known amount of the tritium-labeled toluene standard to the samples, followed by recounting.

RESULTS

Effects of acetylcholine on incorporation of P^{32} into phospholipids of skin. The incorporation of P^{32} into phosphatidic acid, phosphoinositide, phos-

TABLE I

Incorporation of P^{32} into the phosphatides of skin incubated without and with acetylcholine

Condition	No. of patients	Acetyl-choline	Total radioactivities in the phosphatides*			
			Phosphatidic acid	Phosphatidyl inositol	Phosphatidyl choline	Phosphatidyl ethanolamine
			cpm/100 mg fresh tissue			
Controls	7	−	2,120 ± 323	4,810 ± 1,070	8,590 ± 1,930	1,230 ± 328
		+	4,480 ± 660	9,480 ± 1,040	10,800 ± 2,300	1,490 ± 308
Cystic fibrosis of the pancreas	7	−	2,470 ± 450	5,920 ± 1,270	10,300 ± 2,140	1,780 ± 332
		+	4,800 ± 1,170	10,200 ± 2,930	12,800 ± 3,160	1,980 ± 258
Anhidrosis	1	−	2,640	9,250	16,300	2,990
		+	2,910	9,700	12,000	2,230

* The values are averages followed by the standard errors.

TABLE II

Percentage increases in P^{32} incorporation into the phospholipids of skin after incubation with acetylcholine

Condition	Patient	Age	Diagnosis	Increase in radioactivities with acetylcholine: Phosphatidic acid	Phosphoinositide	Phosphatidyl choline	Phosphatidyl-ethanolamine	Sweat Na (mEq/L)
		years		%	%	%	%	%
Controls	P.H.	½	Congenital hydrocephalus	74	56	5	11	23.5
	P.Z.	6	Postpolio deformity	174	282	110	83	11.3
	T.F.	6	Cerebral palsy	122	87	62	103	14.1
	C.R.	6	Astrocytoma	104	158	50	48	12.0
	M.A.	10	Plasma cell hepatitis	124	43	10	26	39.1
	A.S.	10	Undifferentiated mental retardation	160	69	11	−4	20.7
	N.W.	15	Cerebral palsy	66	92	−2	−2	24.0
		Average		117	112	35	38	20.1
Cystic fibrosis	L.B.	¼		45	24	−2	−9	97.0
	P.G.	1½		80	115	63	56	104.3
	B.R.	1½		95	16	−14	−7	92.2
	M.V.	3½		224	140	103	90	114.2
	M.K.	11		76	50	44		142.6
	W.G.	11		64	56	0	−20	108.6
	T.F.	18		44	78	−6	−4	117.8
		Average		90	68	27	15	111.0
Anhidrosis	F.F.	5½	Failure to thrive Increased excretion of dihydroxyphenylalanine	10	5	−20	−25	69.0*

* Value obtained after resumption of sweating.

phatidyl choline, and phosphatidyl ethanolamine are shown in Table I. These values are averages of the total corrected radioactivities of each of these phosphatides from several experiments. The greatest unstimulated incorporation was in phosphatidyl choline, followed by phosphoinositide, phosphatidic acid, and phosphatidyl ethanolamine. The specific activity would of course be greatest in phosphatidic acid, which is present in only minute amounts in tissues (10–12). Phosphatidyl inositol would also be expected to have a higher specific activity than phosphatidyl choline, since its concentration in tissues is very much less than that of phosphatidyl choline.

Acetylcholine approximately doubled the incorporation of P^{32} into phosphatidic acid and phosphatidyl inositol in skin from control children and increased the incorporation of P^{32} into phosphatidyl choline and phosphatidyl ethanolamine 20 to 25%.

With skin from children with cystic fibrosis of the pancreas, the average incorporation of P^{32} into the various phosphatides in the unstimulated tissue and the stimulation by acetylcholine were essentially the same as with skin from control children.

In order to assess the statistical significance of the data, the percentage stimulations of P^{32} incorporation into the phospholipids in each individual experiment were calculated, and these percentage increases were averaged. This was necessitated by the variation in the absolute incorporation of P^{32} into the various phospholipids in the skin from different patients, and would tend to decrease the statistical significance of any differences that might be observed between control and cystic fibrosis skins. The percentage increases in P^{32} incorporation in the various phospholipids in response to acetylcholine and the sodium concentrations in sweat are shown for each patient in Table II. The sodium concentration in the control patients averaged 20.1 mEq per L, (range, 11.3 to 39.1) and in the patients with cystic fibrosis of the pancreas, 111.0 (range, 92.2 to 142.6). This is similar to previous observations [see review (13)]. In control patients, the average percentage stimulations in the various phosphatides were: phosphatidic acid, 117%; phosphatidyl inositol, 112%; phosphatidyl choline, 35%; and phosphatidyl ethanolamine, 38%. In patients with cystic fibrosis of the pancreas, they were: phos-

phatidic acid, 90%; phosphatidyl inositol, 68%; phosphatidyl choline, 27%; and phosphatidyl ethanolamine, 15%. Although the average percentage stimulations were less in patients with cystic fibrosis of the pancreas, the differences were not significant.

Incorporation of P^{32} into phosphatides of skin of a patient with anhidrosis. Of all 15 children in whom incorporation of P^{32} into the phosphatides was studied, only one failed to show a stimulation of P^{32} incorporation into phosphatidic acid and phosphoinositide on incubation of skin with acetylcholine (Tables I and II). One of this child's symptoms was a lack of sweating, and he failed to sweat in response to iontophoresis of pilocarpine into the skin of the forearm. He was malnourished and short, and excreted large amounts of 3,4-dihydroxyphenylalanine in his urine. These findings and a complete case report have been published (14). After a period of adequate nutrition, sweating began. It was considered inadvisable to perform another biopsy at this time because of the development of a keloid at the site of the original biopsy.

The fact that the only child who failed to sweat in response to iontophoresis of pilocarpine into the skin also failed to show a stimulation of P^{32} incorporation into the phosphatides with acetylcholine is compatible with the view that the stimulation of P^{32} incorporation into the phosphatides by acetylcholine is related to the stimulation of sweat secretion by this agent.

Comparison of incorporation of P^{32} and inositol-2-H^3 into phosphoinositide in skin. Previous studies showed that stimulation of P^{32} incorporation into phosphoinositide in pancreas (7), brain cortex (15), and salt gland (2) is accompanied by a stimulation of inositol-2-H^3 incorporation. In brain cortex and salt gland, the percentage stimulation of inositol-2-H^3 incorporation equals the percentage stimulation of P^{32} incorporation. This was also found to be the case in skin (Table III), and is compatible with the view that in these three tissues the inositol phosphate moiety is incorporated as a unit into phosphoinositide on stimulation with acetylcholine.

TABLE III

Comparison of incorporation of P^{32} and inositol-2-H^3 into phosphoinositide

Precursor added	Control	Acetylcholine	Stimulation
	Radioactivity of lipid extract* (total dpm/100 mg tissue)		%
Inositol-2-H^3	3,825	6,770	77
	Radioactivity of phosphatidyl inositol† (cpm/100 mg tissue)		
Orthophosphate-P^{32}	3,980	6,700	69

* Average of duplicates.
† Average of triplicates.

DISCUSSION

The most important finding in this investigation is that stimulation of slices of human skin with acetylcholine, which is the physiological stimulant of sweat secretion, is accompanied by a stimulation of the incorporation of P^{32} into phosphatidic acid and phosphoinositide, with lesser stimulations in phosphatidyl choline and phosphatidyl ethanolamine. Qualitatively, this is very similar to what has been observed on stimulation of secretion in a variety of exocrine and endocrine glands (1, 16).

The secretory segment of the human sweat gland is composed of mucus-secreting cells and clear cells. The latter presumably secrete sodium chloride. Previous work with various glands indicates that the secretion of sodium chloride or of proteins is associated with a phospholipid effect. Stimulation *in vitro* of slices of submaxillary gland—a tissue containing mucus-secreting cells and electrolyte-secreting cells—with cholinergic or adrenergic agents is associated with the secretion of mucin (17) and a phospholipid effect (17, 18). It is probable that electrolyte secretion is also stimulated under these conditions. In interpreting the significance of the phospholipid effect in skin one must therefore bear in mind that the sweat gland contains two types of cells that are probably both responsive to cholinergic stimulation (19). It cannot be ascertained from these studies whether the phospholipid effect is associated with both or only one of these secretory processes in the sweat gland, but in view of the aforementioned studies in other glandular tissues, it seems likely that the phos-

pholipid effect is concerned with both secretory processes. It is hoped that radioautographic studies now in progress with tritium-labeled inositol will throw light on this problem.

Evidence has been obtained that the secretory tubules of the sweat gland secrete a fluid of constant NaCl composition and that NaCl is then partially reabsorbed distally by cells of the collecting duct (20–22). If this is so, the failure to find a difference in the phospholipid response in skin of children with and without cystic fibrosis of the pancreas may not be too surprising, since there are likely to be no differences in the rates of formation of the primary secretion of NaCl. That in the one subject who did not sweat at the time of observation there was no phospholipid effect in the skin is compatible with the view that the phospholipid effect is concerned with the formation of the primary secretion.

It is unfortunate that only one case of anhidrosis was available in this study, since similar results in additional cases would provide further support to a role of phospholipid metabolism in sweat secretion. That acetylcholine, however, which is the physiological trigger for sweat secretion *in vivo* and which would be expected to act in a highly specific manner, produced a phospholipid effect in skin is itself evidence that phospholipid metabolism is associated with sweat secretion.

These studies indicate that the defect in glandular secretion in cystic fibrosis of the pancreas is unlikely to be connected with a gross defect in the metabolism of the phosphatides, as measured by P^{32} incorporation into these phosphatides after 2 hours of incubation.

SUMMARY

Stimulation with acetylcholine of slices of skin from children with and without cystic fibrosis of the pancreas led to a twofold increase in the incorporation of P^{32} into phosphatidic acid and phosphoinositide after 2 hours of incubation. The incorporation of P^{32} into phosphatidyl choline and phosphatidyl ethanolamine was increased to a proportionately lesser extent. There were no statistically significant differences in the phospholipid responses between control skins and skins from patients with cystic fibrosis of the pancreas. In one patient who did not sweat in response to iontophoresis of pilocarpine into the skin, there was no phospholipid effect *in vitro* in response to acetylcholine. These studies provide another example of the relationship between secretion and the metabolism of phosphatidic acid and phosphoinositide.

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