THE EFFECTS OF SECRETAGOGUES ON THE INCORPORATION OF [2-^{3}H]MYOINOSITOL INTO LIPID IN CYTOLOGICAL FRACTIONS IN THE PANCREAS OF THE GUINEA PIG IN VIVO

DOROTHY GERBER, MARGARET DAVIES, and LOWELL E. HOKIN

From the Department of Pharmacology, University of Wisconsin Medical Center, Madison, Wisconsin 53706

ABSTRACT

Stimulation of enzyme secretion in the pancreas on injection of a single dose of the cholinergic drug, pilocarpine, was associated with an increased incorporation of [2-^{3}H]myoinositol into a lipid, which was previously characterized as phosphatidylinositol. Stimulation of enzyme secretion by hourly injection of the pancreozymin congener, caerulein, led to more increased phosphatidylinositol synthesis than with a single injection of pilocarpine. The amylase level of the pancreas remained at a low level as long as caerulein was injected, indicating continued stimulation of enzyme secretion even though increased phosphatidylinositol synthesis ceased after 6 h. Feeding gave the same stimulation of phosphatidylinositol synthesis as caerulein. The major synthesis of phosphatidylinositol in controls and the stimulation of phosphatidylinositol synthesis by pilocarpine was entirely confined to the microsome fraction throughout the experiments (up to 18 h). This shows that there is no flow of microsomal membrane (smooth- or rough-surfaced endoplasmic reticulum) to other membranous structures throughout the secretory cycle and beyond. It is concluded that the stimulation of phosphatidylinositol synthesis by pancreatic secretagogues is confined to microsomal elements and does not play any role in membrane flow.

Previous studies showed that when enzyme secretion was stimulated in pancreas slices incubated with various radioactive phospholipid precursors, there was an increased synthesis of certain specific phospholipids, particularly phosphatidylinositol (see review by L. E. Hokin, 1968). Combined studies utilizing isolation of cellular fractions by differential centrifugation (Redman and Hokin, 1964) and lipid autoradiography (Hokin and Huebner, 1967) with [^{32}P]orthophosphate and [2-^{3}H]myoinositol as precursors, respectively, indicated that the increased synthesis of phosphatidylinositol occurred in the rough-surfaced and smooth-surfaced endoplasmic reticulum (Golgi apparatus?).

One hypothesis which has been proposed to explain the increased synthesis of phosphatidylinositol in the smooth- and rough-surfaced endoplasmic reticulum is based on the postulate of membrane flow accompanying intracellular transport of zymogens. This hypothesis has been reviewed in some detail elsewhere (L. E. Hokin, 1968). Arguments against it have also been reviewed (Hokin, 1969).

If the above hypothesis were correct, one would expect to see the stimulated incorporation

of [2-^{3}H]myoinositol into phosphatidylinositol moving with time from microsomal elements to the zymogen granules. Previous studies showed that 2 h after stimulation of pigeon pancreas slices with acetylcholine (ACh) there was very little stimulation of ^{32}P incorporation into the zymogen granules. However, in vitro studies suffer from the fact that the stimulation of secretion is small in comparison to what can be achieved in vivo, and the viability of the pancreas is limited so that longer term experiments cannot be performed.

In the present study the incorporation of [2-^{3}H]-myoinositol into lipid (which has been shown to be a good measure of incorporation into phosphatidylinositol in the guinea pig [Hokin and Huebner, 1967]) has been followed for long periods of time in guinea pig pancreas after a single pulse of [2-^{3}H]myoinositol by intraperitoneal injection. The incorporation of [2-^{3}H]myoinositol has been followed in pancreases of fasted control animals, animals which have received a single dose of pilocarpine, animals which have received hourly injections of caerulein up to 24 h, or animals which have been given food ad lib. after fasting. Various cytological fractions have been isolated, as well as a purified zymogen granule fraction, and the increases in [2-^{3}H]myoinositol incorporation into lipid in these fractions have been followed.

MATERIALS AND METHODS

Materials

Guinea pigs were obtained from Marvin O'Brien, Oregon, Wis., from a mixed population. They were provided with a constant supply of Purina Guinea Pig Chow (Ralston Purina Co., St. Louis, Mo.) and water until 12 h before the experiment, when the food was removed from the cage.

[2-^{3}H]Myoinositol was prepared and purified as described previously (Hokin, 1965), except that carrier myoinositol was added before crystallization. The myoinositol was assayed by the Wisconsin Alumni Research Foundation by the method of Atkin et al. (1943). Pilocarpine nitrate, obtained from Sigma Chemical Co., St. Louis, Mo., and the [2-^{3}H]myoinositol were diluted in isotonic 0.9% NaCl before injecting. A sample of caerulein was a kind gift of Dr. Morton I. Grossman, Veterans Administration Center, Los Angeles, Calif. Subsequently, synthetic caerulein, B grade, was obtained from Calbiochem, Los Angeles, Calif., and diluted for use in isotonic NaCl. Butylated hydroxytoluene (BHT) was obtained from the Hercules Incorporated, Wilmington, Del. The Amersham/Searle Corp., Arlington Heights, Ill., provided the Nuclear Chicago Solubilizer (NCS) and the Spectrafluor 2,5-diphenyloxazole-1,4-bis-[2-(5-phenyloxazolyl)]benzene (PPO-POPOP) concentrated scintillation fluid. All other chemicals were reagent grade.

Preparation of Animals

Male guinea pigs weighing 300–600 g were used. After 12 h of fasting they were injected with 66 μCi/kg body weight of [2-^{3}H]myoinositol (sp act, 16 mCi/mmol). At the same time the stimulated animals were injected with 10 mg/kg pilocarpine nitrate or 1 μg/kg caerulein. The injections of caerulein were repeated hourly. All injections were given intraperitoneally. Other animals were given food ad lib.

At times from 1 to 48 h after injection the guinea pigs were killed by a blow on the head followed by decapitation. A 2–4 ml sample of blood was obtained from the severed vessels in the neck, allowed to clot in a test tube, and the serum was separated by centrifugation at 3,000–4,000 rpm for 10 min in an International refrigerated centrifuge (International Equipment Co., Needham Heights, Mass.).

Homogenization

The pancreas was removed and trimmed of connective tissue and weighed as quickly as possible. It was then cut up with fine scissors into small pieces and homogenized in a solution containing 0.32 M sucrose and 0.01 M NaF. In this and all subsequent stages the tissue was kept at 0°–4°C, or if storage for periods longer than a few hours was required before protein assay, the tissue homogenate was frozen and stored at −20°C. This was found not to affect the protein assay, even after a few months had elapsed. The homogenization was carried out in a Brendler-type homogenizer, size C, from Arthur H. Thomas Co., Philadelphia, Pa., with a glass mortar and Teflon pestles, with a clearance of 0.04 inch for the first stage, and 0.006–0.009 inch for a second homogenization. The pestles were driven at approximately 3,000 rpm by a Tri-R Stir-R motor (Tri-R Instruments, Inc., Rockville Centre, N. Y.). The ratio of homogenizing solution to tissue weight was kept constant at 10 ml/g tissue. The homogenate was filtered through nonwoven cloth (Miracloth; Chicopee Mills, Inc., New York), the homogenizer was rinsed with an additional 1–2 ml of medium, and the volume of the homogenate was measured in a small graduated cylinder.

Aliquots of the homogenate were taken for assay of total radioactivity, lipid radioactivity, phospholipid phosphorus (phospholipid-P), and protein.

Subcellular Fractionation

Differential centrifugation was accomplished essentially by the method of Jamieson and Palade (1966). In some experiments a variant of this procedure was used. All particulate material was first sedimented at 105,000 *g* for 45 min, the pellet was rehomogenized, and the nuclei, zymogen granules, and mitochondria were sedimented by the method of Jamieson and Palade (1966). The supernatant contained the microsomes only. This procedure was used to separate as quickly as possible the soluble enzymes of the cell which are responsible for breakdown of phospholipids. The first method resulted in a loss of 30–60% of the radioactivity in the lipid extract during differential centrifugation, as measured by summing the radioactivity in the various fractions and comparing it with that in the original homogenate. The second method reduced the loss to 15–30%.

Isolation of Purified Zymogen Granules

In some experiments the zymogen granule fraction was purified by the method of Meldolesi et al. (1971). Samples of this zymogen granule fraction were examined by electron microscopy and found to be only minimally contaminated with other cell organelles.

Separation of Smooth and Rough Microsomes

Smooth and rough microsomes from 1 ml of microsomal suspension were separated on a sucrose density gradient by a method adapted from that of Jamieson and Palade (1967). Cellulose nitrate tubes with a volume of 14.2 ml were centrifuged in a Spinco SW 40 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 198,800 *g* for 300 min.

Lipid Extraction

After differential centrifugation all fractions were suspended in 5 ml of the sucrose-fluoride medium by homogenization. 2–4 ml aliquots were added to equal volumes of cold 10 or 16% TCA, and the lipids were extracted, as described previously (Hokin and Hokin, 1959), except that the amount of chloroform ethanol was adjusted to give 1 ml of solvent/3 mg wet weight of original tissue. This ratio was established by plotting the radioactivity in the lipid extract vs. the milligrams of tissue in the homogenate. The recovery of radioactivity fell off gradually above 3 mg of tissue/ml of extracting medium. The addition of about 1 mg/ml BHT to the chloroform-ethanol resulted in a better recovery of lipid, presumably by preventing peroxidation. An aliquot of the chloroform phase containing the lipids was transferred to a polyethylene scintillation vial and was dried under a stream of nitrogen at about 40°C. Residual TCA was then removed by placing the vials in a vacuum desiccator containing KOH and evacuating for 2 h.

Determination of Radioactivity

Toluene-PPO-POPOP scintillation fluid was added to the vials, and the radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer, Model 3380 (Packard Instrument Co., Downers Grove, Ill.), which had been calibrated with standard solutions so that, from the automatic external standardization (AES) channel, quenching of the sample could be corrected for, and an accurate estimate of the actual number of disintegrations per minute (dpm) could be made. The total radioactivity in the tissue was determined by dissolving overnight an aliquot (0.1 ml) of the homogenate in 1 ml of NCS in a scintillation vial, adding scintillation fluid, and determining radioactivity, as described above. Radioactivity in the serum was determined in a similar manner.

Assays

Protein in all fractions was determined in duplicate by the method of Lowry et al. (1951). Bovine serum albumin, either crystalline or a sterile solution from ICN Nutritional Biochemicals Div., International Chemical and Nuclear Corp., Cleveland, Ohio, was used as a standard. Phospholipid-P in chloroform extracts was determined by the method of Bartlett (1959). All values were corrected to a constant wet weight of pancreas. The weight of the pancreas was shown not to alter significantly on stimulation (Table I). Disintegrations per minute in the various samples were corrected to a constant tissue radioactivity determined by counting the NCS-solubilized ho-

TABLE I

Effect of Stimulation of Enzyme Secretion by Repeated Injection of Caerulein on Pancreatic Wet Weight, Phospholipid, and Protein

	Unstimulated			Stimulated		
Time	Weight	PLP-P	Protein	Weight	PLP-P	Protein
h	*mg*	*μg P/g*	*mg/g*	*mg*	*μg P/g*	*mg/g*
1	1,000	782	130	962	776	135
3	920	760	154	965	793	102
6	850	714	119	911	847	149
9				909	798	145
12	765	785	157	916	779	135

Phospholipid phosphorus and protein (PLP-P) were determined as described under Materials and Methods.

mogenate. Amylase assays were carried out as described previously (Hokin, 1951).

Paper Chromatography of Deproteinized Serum

Serum was deproteinized by adding 1 part of 25% perchloric acid to 4 parts of serum at 0°C, centrifuging at 0°C, neutralizing the supernatant with 1 N KOH at 0°C, and removing the potassium perchlorate by centrifugation at 0°C. An aliquot of the supernatant was spotted on Whatman no. 1 paper with appropriate standards, and ascending chromatography was carried out in propanol/ethanol/water (100:60:40). The spots were detected with ammoniacal silver nitrate.

Statistical Analysis

The data were averaged, and the standard error of the mean was calculated. The means of two samples were compared using Student's *t* test.

RESULTS

Effect of Pilocarpine on [2-^{3}H]Myoinositol Incorporation into Total Cell Lipid

Fig. 1 shows the incorporation of [2-^{3}H]myoinositol into lipid in the homogenates of pilocarpine-stimulated and control pancreases with time up to 24 h. Statistically significant increases in radioactivity were observed from 1 to 12 h after a single injection of pilocarpine. By 24 h the radioactivity in the stimulated tissue had returned to the control value. The increased rate of phosphatidylinositol synthesis occurred only during the first few hours and roughly paralleled the pharmacological effect of pilocarpine, as evidenced by increased salivation, etc. However, the newly synthesized phosphatidylinositol in response to pilocarpine stimulation remained for many hours after the acute pharmacological effect of pilocarpine had disappeared, indicating that once the phosphatidylinositol was synthesized in response to the pancreatic secretagogue it was slowly degraded.

Effect of Hourly Injection of Caerulein on [2-^{3}H]Myoinositol Incorporation into Phosphatidylinositol

It would be of interest to see how long the pancreas could respond with increased phosphatidylinositol synthesis by maintaining constant stimulation of pancreatic secretion. The toxicity of pilocarpine precluded its use for this purpose. However, caerulein, which resembles pancreozymin in its pharmacological activity, is essentially nontoxic in doses which give good stimulations of pancreatic secretion. Guinea pigs were therefore stimulated at hourly intervals with caerulein up to 24 h. There was a marked increase in the rate of incorporation of [2-^{3}H]myoinositol into lipid with hourly injections of caerulein (Fig. 2) up to 6 h. After this time the rate of incorporation of [2-^{3}H]myoinositol slowly fell to that of the control in spite of constant stimulation by caerulein. These results indicate that a compartment of phosphatidylinositol is synthesized in response to the secretagogues, which does not mix with the

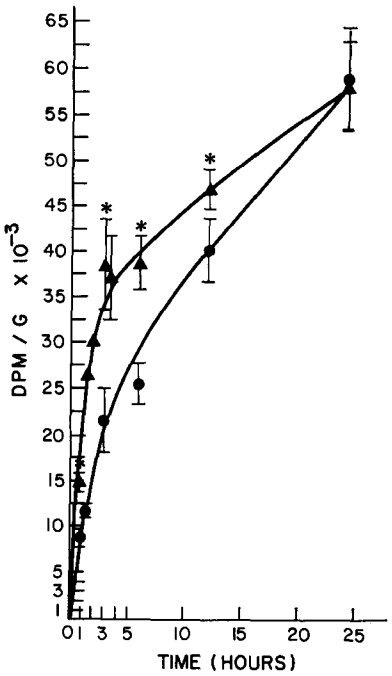

FIGURE 1 Effect of pilocarpine on the incorporation of [2-^{3}H]myoinositol into the lipids of the pancreas. Pilocarpine and [2-^{3}H]myoinositol were injected at zero time, as described under Materials and Methods. The triangles are the lipid radioactivity from pancreases of guinea pigs injected with pilocarpine. The circles are the lipid radioactivity from unstimulated animals. The values are given as the means ± the standard error of the mean. The asterisks indicate that the differences between the unstimulated and stimulated values were significant at the $P < 0.001$ level, as determined by the *t* test.

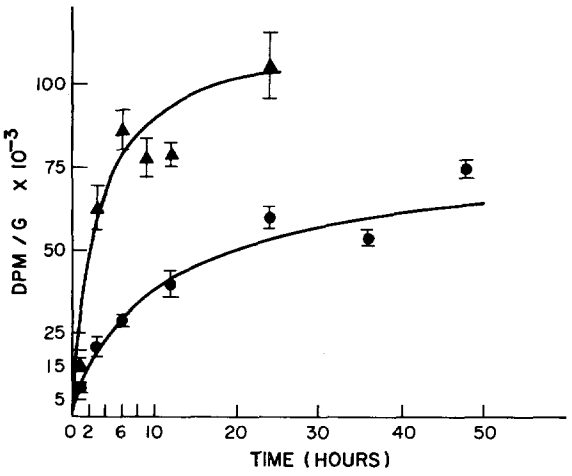

FIGURE 2 Effect of caerulein on the incorporation of [2-^{3}H]myoinositol into the lipids of the pancreas. Caerulein and [2-^{3}H]myoinositol were injected at hourly intervals, as indicated under Materials and Methods. The triangles are the lipid radioactivity from pancreases of guinea pigs injected with caerulein. The circles are the lipid radioactivity from unstimulated animals. The values are given as the means ± the standard error of the mean.

rest of the phosphatidylinositol in the cell. It appears to remain as long as the caerulein is in contact with the acinar cells.

The mean phospholipid-P, protein, and tissue weight were followed with time in controls and in caerulein-stimulated pancreases. There were no significant changes in these parameters either with time or with caerulein stimulation (Table I).

Fig. 3 shows the mean amylase values in the pancreases of control and caerulein-stimulated animals. It can be seen that within 1 h the mean amylase content of the acini had fallen to less than half and continued to fall at a slower rate up to 12 h. Since there is evidence that stimulation of pancreas in vivo with pancreozymin or its congeners stimulates rather than inhibits amylase synthesis (Reggio et al., 1971), it appears likely that the low steady-state level of amylase in the pancreas was due to continuous secretion. This is of considerable interest in connection with the stimulation of phosphatidylinositol synthesis by caerulein since phosphatidylinositol synthesis showed no further increases after 6 h, as compared to the control. This may be taken as further evidence for a dissociation of the phosphatidylinsitol effect from secretion (L. E. Hokin, 1966, 1969; M. R. Hokin, 1968). However, an alternative explanation is that the rate of phosphatidylinositol synthesis was still increased, but it was matched after 6 h by an equally increased rate of phosphatidylinositol degradation.

Comparison of the Effects of Feeding and Chemical Stimuli on the Incorporation of [2-^{3}H]Myoinositol into Lipid

Feeding is the physiological stimulus for pancreatic enzyme secretion and is mediated via vagal (acetylcholine) and humoral (pancreozymin) pathways. It was therefore of interest to compare feeding with stimulation by pilocarpine and caerulein. Four groups of animals were fasted for 12 h and then injected with [2-^{3}H]myoinositol, as described under Materials and Methods. The animals were given either a single dose of 10 mg/kg pilocarpine, 1 μg/kg caerulein at hourly intervals, or Purina Chow ad lib. The controls were continued on fast. 12 h later the animals were killed and the radioactivity in the lipid was determined, as described under Materials and Methods. The results are shown in Table II. The stimulation of [2-^{3}H]myoinositol incorporation into lipid in response to feeding was very similar to that seen with hourly injections of caerulein.

Earlier times were tried with the feeding experiments but were unsuccessful because the guinea pigs were disturbed by the injection of [2-^{3}H]myoinositol and did not resume normal feeding for up to 3 h.

Turnover of Phosphatidylinositol in Pancreas

Table III shows the turnover of phosphatidylinositol in pancreases from control and caerulein-

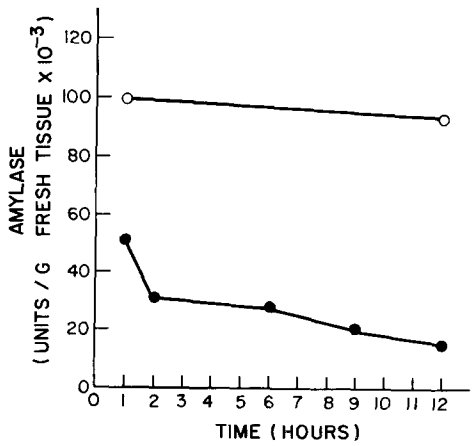

FIGURE 3 Effect of caerulein on the amylase content of guinea pig pancreas. Caerulein was injected at hourly intervals, as described under Materials and Methods. The open circles are the amylase levels from control animals. The solid circles are the amylase levels from the animals stimulated with caerulein. Amylase content was assayed, as described under Materials and Methods.

TABLE II

Effects of Various Forms of Stimulation of Enzyme Secretion on the Incorporation of [2-^{3}H]-Myoinositol into Lipid in the Pancreas of the Guinea Pig

Condition	Lipid radioactivity
	dpm/g fresh tissue
Fasting	30,800 ± 8,490*
Pilocarpine	49,400 ± 11,400
Fed	80,100 ± 22,600
Caerulein	79,000 ± 3,580

The modes of stimulation of enzyme secretion are described in the text. The animals were killed 12 h after initiation of stimulation.The lipid radioactivity is corrected to 360,000 dpm/g tissue for the whole tissue homogenate.

* Standard error of the mean.

stimulated animals. In control animals at 12 h 13% of the total tissue phosphatidylinositol had turned over. In caerulein-stimulated animals 29% of the phosphatidylinositol had turned over. These calculations are based on a value of 3.28 μmol of free inositol/g wet weight of guinea pig pancreas, as determined by Dawson and Freinkel (1961), and 3.07 μmol phosphatidylinositol/g wet weight of rat pancreas, as determined by Dittmer and Douglas (1969). The data in Figs. 1 and 2 suggest that there are several compartments of phosphatidylinositol with different turnover rates. In the

TABLE III

Phosphatidylinositol Turnover in the Presence and Absence of Caerulein Stimulation of the Pancreas

	Unstimulated		Stimulated	
Time	PI synthesized	Turnover	PI synthesized	Turnover
h	*μmol/g tissue*	%	*μmol/g tissue*	%
1	0.068	2.22	0.150	4.86
3	0.178	5.80	0.664	21.6
6	0.276	8.98	1.047	34.1
9			0.890	29.1
12	0.401	13.1	0.904	29.4

The percent of phosphatidylinositol turned over was calculated as described in the text. PI = phosphatidylinositol.

control animals there appears to be a compartment with a fairly fast turnover rate, as indicated by the rapid initial incorporation, and at least one compartment with a slower turnover rate, as indicated by the slower incorporation after about 8 h. As discussed above, the fraction of phosphatidylinositol which incorporates [2-^{3}H]myoinisitol in response to secretagogues appears to be yet another compartment. Akino and Shimojo (1970) have found different turnover rates for phosphatidylinositols with different fatty acid substituents.

Effect of Pilocarpine on the Incorporation of [2-^{3}H]Myoinositol into Subcellular Fractions

Fig. 4 shows the incorporation of [2-^{3}H]myoinositol into the lipid of the various cellular fractions. The only fraction which showed appreciable incorporation up to 18 h was the microsome fraction, as was previously shown with pancreas slices incubated for shorter periods in vitro with ^{32}P (Redman and Hokin, 1964). The only significant stimulations of [2-^{3}H]myoinositol incorporation were in the microsomal fraction and in the nuclear fraction at 3 h. All other differences between means of stimulated and control samples were not significant at the 5% level.

The stimulation of [2-^{3}H]myoinositol incorporation into lipid in the homogenate must therefore reflect stimulation of incorporation in the microsomal fraction. The statistically significant stimulation of incorporation in the nuclear fraction at 3 h probably represents whole cell contamination.

When the zymogen granules were isolated by the technique of Meldolesi et al. (1971), there was still no stimulation of [2-^{3}H]myoinositol in-

corporation into lipid. A few experiments were also carried out using hourly stimulation with caerulein followed by isolation of the zymogen granules by the technique of Meldolesi et al. (1971). No significant differences in radioactivity between stimulated and control zymogen granules were seen.

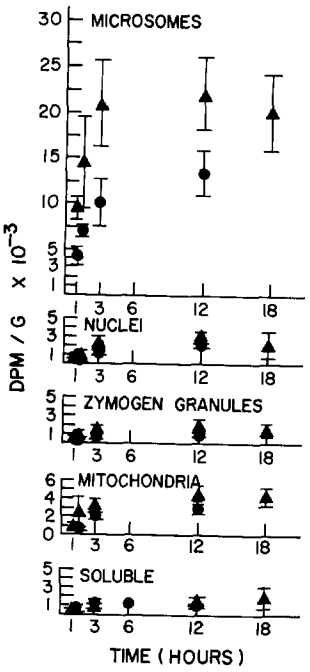

FIGURE 4 Effect of pilocarpine on the incorporation of [2-^{3}H]myoinositol into lipid in various cellular fractions of the guinea pig pancreas. The triangles are radioactivity from pilocarpine-stimulated animals, and the circles are radioactivity from control animals. The vertical bars are the standard error of the mean.

Several attempts were made to separate the smooth and rough microsomes on a sucrose density gradient by the method of Jamieson and Palade (1967). Bands corresponding to the smooth and rough microsomes described by Jamieson and Palade (1967) were observed on the gradients. Analysis of radioactivity in the phospholipids in these fractions showed stimulations in both the smooth and rough microsomes, but because of the extensive breakdown of phospholipid-inositol radioactivity (30–60%) due to the prolonged centrifugation time, a quantitative comparison of stimulations in each fraction cannot be made. However, qualitatively these results confirm the earlier autoradiographic studies of Hokin and Huebner (1967) which showed a stimulation in regions of the cell occupied almost exclusively by rough-surfaced endoplasmic reticulum on the one hand and by smooth-surfaced endoplasmic reticulum on the other hand.

Table IV shows that there was no significant change in the total phospholipid-P in the zymogen granule fraction isolated in the usual way after stimulation with pilocarpine. Similar findings were obtained if a more purified zymogen granule fraction was obtained by the method of Meldolesi et al. (1971). These findings indicate that the total membrane isolated in the zymogen granule fraction was still recovered in essentially the same amount on stimulation. These observations validate the method of expressing radioactivity in the fractions, as disintegrations per minute per gram fresh tissue. A similar lack of effect of philocarpine on the total phospholipid-P was found in the other fractions. For example, the microsome fraction, which contained the bulk of the phospholipid-P, contained 450 μg phospholipid-P/g fresh

TABLE IV

Lack of Effect of Pilocarpine on the Phospholipid Content of the Zymogen Granule Fraction in Pancreas

	Phospholipid-P (μg P/g)						
	Unstimulated			Stimulated			
Time	Mean	Range	No. exps.	Mean	Range	No. exps.	P value
h							
1	35	21–51	5	29	24–32	5	0.36
3	47	12–75	7	44	20–52	7	0.42
12	42	19–63	6	51	10–114	6	0.59

The zymogen granules were isolated by the standard purification procedure. μg P/g refers to micrograms phospholipid-P per gram of fresh tissue.

tissue, and this value did not change with time or with stimulation.

Serum Radioactivity

Fig. 5 shows the changes in serum radioactivity up to 24 h after a single injection of [2-^{3}H]myoinositol. There was a rapid fall in radioactivity between the 1st and 3rd h after injection to about half the initial level, after which the radioactivity fell very slowly over the next 21 h. Injection of pilocarpine or caerulein did not affect the serum radioactivity. Paper chromatography showed that almost all of the radioactivity in the serum was in free myoinositol with traces in a spot with the same R_f as glucose. The rapid initial fall in serum radioactivity may be due to uptake into the tissues, but this is not indicated by the tissue level of radioactivity in the pancreas since the radioactivity in this tissue had already reached a maximum at 1 h. Uptake in other tissues may be slower, however. Lipid extraction of TCA-precipitated serum indicated that no more than 1% of the serum radioactivity was in lipid.

Tissue Radioactivity

The total radioactivity in the tissue averaged 360,000 dpm/g tissue, was not affected by stimulation of secretion, and did not change significantly over a 48 h period (Table V). All lipid radioactivity was accordingly corrected to a constant value of 360,000 dpm/g tissue to correct for variations in tissue radioactivity due to some variability in the amount of [2-^{3}H]myoinositol delivered by injection intraperitoneally. A maximum of 15% of the total pancreatic radioactivity was lipid, the remainder presumably being free myoinositol. Thus, the level of myoinositol in the tissue was 6–7 times that in serum, indicating concentrative uptake of inositol by the pancreas. This was previously shown for ascites cells incubated in vitro (Johnstone and Sung, 1967).

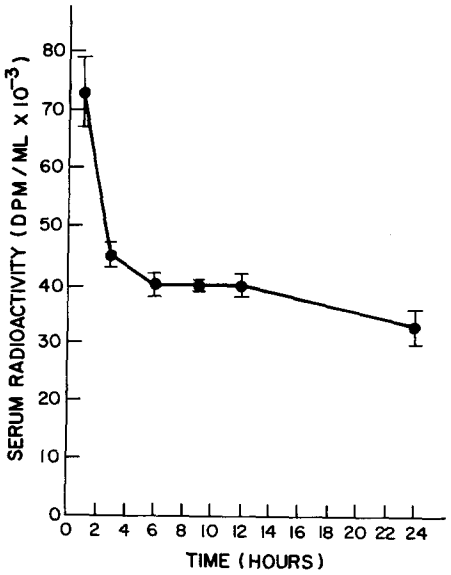

FIGURE 5 The radioactivity in guinea pig serum after injection of [2-^{3}H]myoinositol. The vertical bars represent the standard error of the mean. The radioactivity in the serum was determined, as described under Materials and Methods.

TABLE V

Radioactivity in Homogenate of Pancreas after Injection of [2-^{3}H]Myoinositol with and without Caerulein

Time	Unstimulated	Stimulated
h	*dpm/g*	*dpm/g*
1	360,000 ± 10,800*	325,000 ± 30,900
3	362,000 ± 44,700	305,000 ± 19,000
6	314,000 ± 18,000	278,000 ± 12,300
9		260,000 ± 15,900
12	355,000 ± 17,500	263,000 ± 21,600
24	338,000 ± 16,700	
36	317,000 ± 9,200	
48	273,000 ± 16,700	

Radioactivity in the homogenate was determined as described under Materials and Methods.
* Standard error of the mean.

DISCUSSION

Stimulation of pancreatic secretion in guinea pigs by injection of the cholinergic drug, pilocarpine, or the polypeptide hormone, caerulein, which is a potent analogue of pancreozymin, led to an increased rate of incorporation of [2-^{3}H]myoinositol into lipid. Previous in vitro studies showed that phosphatidylinositol was responsible for essentially all of the radioactivity incorporated into the lipid extract from [2-^{3}H]myoinositol in pancreas. Feeding led to a stimulation of [2-^{3}H]myoinositol incorporation comparable to that achieved by hourly injection of caerulein. With the single dose of pilocarpine the increased rate of phosphatidylinositol synthesis lasted for 2 h; after 22 h the newly synthesized phosphatidylinositol had disappeared. With repeated injections of caerulein the rate of phosphatidylinositol synthesis increased

for 6 h. After this time, in spite of hourly injections of caerulein, the level of phosphatidylinositol radioactivity paralleled that of the control. Amylase secretion, as measured by tissue levels of amylase, did not diminish over a period of 12 h. Thus, amylase secretion would not appear to be dependent on an increased rate of synthesis of phosphatidylinositol unless phosphatidylinositol degradation was stimulated by caerulein so that it matched phosphatidylinositol synthesis by 6 h. Other lines of evidence have previously been presented which show a dissociation of enzyme secretion from the phosphatidylinositol effect in pancreas in vitro. If calcium is omitted from the incubation medium, amylase secretion in response to acetylcholine is blocked, but about three-quarters of the full phosphatidylinositol effect is retained (Hokin, 1966). Amylase secretion and the phosphatidylinositol effect do not show a close parallelism when the concentration of secretagogue is varied (M. R. Hokin, 1968) or if one follows the two processes with time (M. R. Hokin, 1968). It would thus appear that the phosphatidylinositol effect and enzyme secretion are two independent effects of secretagogues.

There is good evidence that after the secretory proteins are synthesized on the cytosol side of the rough-surfaced endoplasmic reticulum they are transported to the cisternal spaces (Palade et al., 1962). The secretory proteins then move by a mechanism, which is as yet unclear, from the cisternal spaces to the Golgi apparatus and from there to prozymogen granules, finally ending up in mature zymogen granules. One mechanism which has been suggested for intracellular transport of secretory proteins is a budding of membrane from the rough-surfaced endoplasmic reticulum to form the membranes of prozymogen granules. After coalescence of zymogen granule membrane with the plasma membrane by exocytosis the excess membrane or its constituents are released into the cytoplasm and are utilized for reassembly of rough-surfaced endoplasmic reticulum. The increased incorporation of [2-^{3}H]myoinositol into phosphatidylinositol in response to secretagogues serves as a marker for microsomal membrane. If the above postulated movement of membrane did in fact occur, one would expect to see a flow of radioactivity from the microsomes to the zymogen granules after stimulation with pilocarpine or caerulein. This in fact did not occur. The radioactivity in the zymogen granule fraction remained very low and at no time over the 24 h time period did it show an appreciable increase in response to either pilocarpine or caerulein. In fact, there was no evidence for radioactivity being transferred from the microsomes to any fraction over the 24 h time period. These data strongly suggest that the increased synthesis of phosphatidylinositol in response to secretagogues is confined to the microsomal fraction at all times and plays some role in microsomal function. This is consonant with the above discussion which indicates that the phosphatidylinositol effect and enzyme secretion are two independent responses to pancreatic secretagogues.

It was quite surprising to find that the free inositol radioactivity in the pancreas remained quite constant over such prolonged periods. This precluded analysis of the flow of radioactivity from microsomes to other membrane fractions by the usual pulse-labeling type of experiment. However, if there were transfer of stimulated radioactivity from the microsome fraction to any other fraction with time, the stimulated radioactivity would have been seen in other membrane fractions with time.

The fact that the phosphatidylinositol effect is elicited by quite different types of secretagogues which lead to one final common pathway, namely, enzyme secretion, suggests that the phosphatidylinositol effect is related in some way to enzyme secretion. One possibility which has been suggested is that it is an adaptive response to the secretagogue, i.e., when secretagogue constantly impinges on the acinar cell, the newly synthesized phosphatidylinositol in the rough and smooth endoplasmic reticulum "gears up" the endoplasmic reticulum for increased synthetic and secretory work (M. R. Hokin, 1968). However, this "gearing up" would not depend on a continued increased rate of phosphatidylinositol synthesis since the latter stopped after 6 h of continued injection of caerulein (this assumes that it was not matched by an equivalent increased degradation of the same phosphatidylinositol molecules which had been newly synthesized). Yet the cell was obviously still responding to caerulein, as measured by its amylase levels.

The physiological significance of the "phospholipid effect," which has been studied in a variety of glands and in nervous tissue over a period of 20 yr since its discovery, is thus still not clear.

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