RADIOAUTOGRAPHIC LOCALIZATION OF THE INCREASED SYNTHESIS OF PHOSPHATIDYLINOSITOL IN RESPONSE TO PANCREOZYMIN OR ACETYLCHOLINE IN GUINEA PIG PANCREAS SLICES

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ABSTRACT

A technique is described for measuring the incorporation of *myo*-inositol-2-^{3}H into the lipid of various regions of the guinea pig pancreatic acinar cell by radioautography. Stimulation of enzyme secretion with either pancreozymin or acetylcholine was associated with increased graining in both the basophilic cytoplasm and the nonbasophilic cytoplasm. Kinetic studies suggested that the incorporation of *myo*-inositol-2-^{3}H was stimulated independently in the two regions. Most of the increment in graining due to stimulation with pancreozymin or acetylcholine plus eserine was abolished if the tissue was extracted with 2:1 chloroform-methanol before radioautography. On chromatography of lipid extracts of pancreas, the only lipid showing a detectable increment in radioactivity on stimulation with pancreozymin was phosphatidylinositol. Thus, essentially all of the increment in graining is likely to be due to increased incorporation of tritium into phosphatidylinositol. These studies, coupled with earlier studies employing differential centrifugation, indicate that on stimulation of enzyme secretion there is increased synthesis of phosphatidylinositol in the rough-surfaced endoplasmic reticulum and in the smooth-surfaced Golgi membranes. The significance of these observations is discussed in connection with membrane circulation presumed to occur in the pancreatic acinar cell on stimulation of protein secretion. It is suggested that the increased synthesis of phosphatidylinositol may be concerned with the formation of new endoplasmic reticulum and possibly Golgi membrane to replace that which is presumably converted to membrane of the zymogen granules during intracellular protein transport.

When pigeon pancreas slices are stimulated to secrete protein by incubating with either acetylcholine (ACh) or pancreozymin, there is a marked stimulation of the synthesis of phosphatidylinositol, as evidenced by the increased incorporation of ^{32}P, glycerol-1-^{14}C, and inositol-2-^{3}H into this lipid (1). Separation of various cellular fractions by differential centrifugation reveals that the increased synthesis of phosphatidylinositol is primarily in the membranous components of the microsome fraction (2). Recent studies by Jamieson and Palade (3) show that the pancreatic microsome fraction consists of smooth-surfaced and rough-surfaced vesicles derived from the Golgi complex and the endoplasmic reticulum, respectively. The pancreatic exocrine cell lends itself rather well to studies of these two types of membranes by radioautographic techniques, since the smooth-surfaced Golgi membranes are located almost exclusively in the supranuclear or centrosphere region of the cell and the rough-surfaced endoplasmic reticulum is located predominantly in the basal part of the cell.

In this paper, a technique is reported in which

the incorporation of tritium from *myo*-inositol-2-^{3}H into phosphatidylinositol is followed by radioautography. Grain counts in various regions of the cell indicate that the increased incorporation of *myo*-inositol-2-^{3}H into phosphatidylinositol occurs in the regions of both the Golgi membranes and the rough-surfaced endoplasmic reticulum at times as short as 2–5 min after stimulation with ACh. The kinetic curves of incorporation of tritium in both regions suggest that the increased incorporation of inositol-2-^{3}H into phosphatidylinositol may be a primary effect in both rough and smooth membranes. The physiological significance of the phosphatidylinositol effect for membrane circulation during protein secretion is discussed in the light of these observations. Part of this work has been presented in preliminary form at Symposia (4–6).

MATERIALS AND METHODS

Incubation

Adult guinea pigs were sacrificed by a blow on the head, and the pancreas was quickly removed and placed in ice-cold 0.9% NaCl. Slices were then prepared with a Stadie-Riggs (7) microtome and placed in a covered crystallizing dish sitting on ice. Surface slices were rejected. The slices were then weighed in a torsion balance and incubated in 25-ml Erlenmeyer flasks as described in the Tables and Figures.

Estimation of Lipid Radioactivity

Total lipid radioactivity was determined as described previously (8). The chromatographic separation of phosphoinositides and strip counting of the chromatogram have also been described (8).

Materials

myo-Inositol-2-^{3}H was prepared and purified as described previously (1, 8). It gave a single radioactive peak on chromatography in propanol-ethanol-water (100:60:40). Pancreozymin was a commercial preparation termed Cecekin, which was obtained from Vitrum, Stockholm. Acetylcholine chloride was obtained from Matheson, Coleman, and Bell, Cincinnati, Ohio. Eserine sulfate was obtained from Nutritional Biochemical Corp., Cleveland. The carbowax preparation consisted of 80 ml of carbowax 1,000, 20 ml of carbowax 4,000, both melted and filtered, and 1.22 g of carbowax 20,000. The carbowaxes were obtained from Carbide and Carbon Chemicals Co., New York. Standard phosphatidylinositol was prepared as described previously (9).

Radioautographic Technique

Tissue for radioautography was fixed in 10% neutral formalin for 24 hr at room temperature. After fixation, we rinsed the tissue for another 24 hr in running tap water to remove excess formalin and any remaining water-soluble radioactivity. Embedding was carried out in a vacuum oven at 50–52°. The tissues were suspended in a 1:1 mixture of carbowax-water for 20 min. They were transferred to the undiluted carbowax preparation, where they remained for 90 min or overnight. They were embedded in carbowax in 2-in-square paper boxes. The molten carbowax was poured into the boxes, and as soon as cloudiness appeared the tissue was dropped in without stirring. We left the boxes undisturbed for at least 30 min to allow hardening, and they were not sectioned until at least 2 hr had elapsed. For sectioning, the cardboard box was removed, and the block was cut down with a razor and mounted on a block holder. Blocks were stored in a desiccator over calcium chloride. The tissue blocks were sectioned with a rotary microtome in an air-conditioned room (20°) having a relative humidity no higher than 60%. Sections were 2μ thick. The sections were floated on a thin film of gelatin dichromate affixative which was placed directly on an acid-cleaned air-dried slide. The slides were then dried at room temperature for 24 hr. After drying, we removed the embedding mixture by placing the slides in distilled water for 5–10 min. They were then drained and allowed to dry. Radioautograms were prepared according to the stripping film technique of Doniach and Pelc (10). The exposure time was 28 days. The slides were stored at 4° during exposure. After exposure, they were developed for 10 min in Kodak D 19B developer, rinsed once or twice in distilled water, and fixed for 10–12 min in Kodak Fixer. They were rinsed for ten 5-min periods in fresh changes of tap water and finally twice in distilled water. They were then air dried. We stained the tissues through the film by placing the slides in distilled water for 3 min and then in 0.5% toluidine blue for 30 min. They were then rinsed in distilled water and drained dry. The slides were mounted with "Permount."

Extraction of Tissues Sections with Chloroform-Methanol

Slides containing tissue sections were submerged in 2:1 chloroform-methanol for 1 hr and then submerged in fresh chloroform-methanol for an additional hour. They were then washed slowly for 1 hr in running tap water. They were finally air dried at room temperature. Stripping film radioautography was carried out as usual.

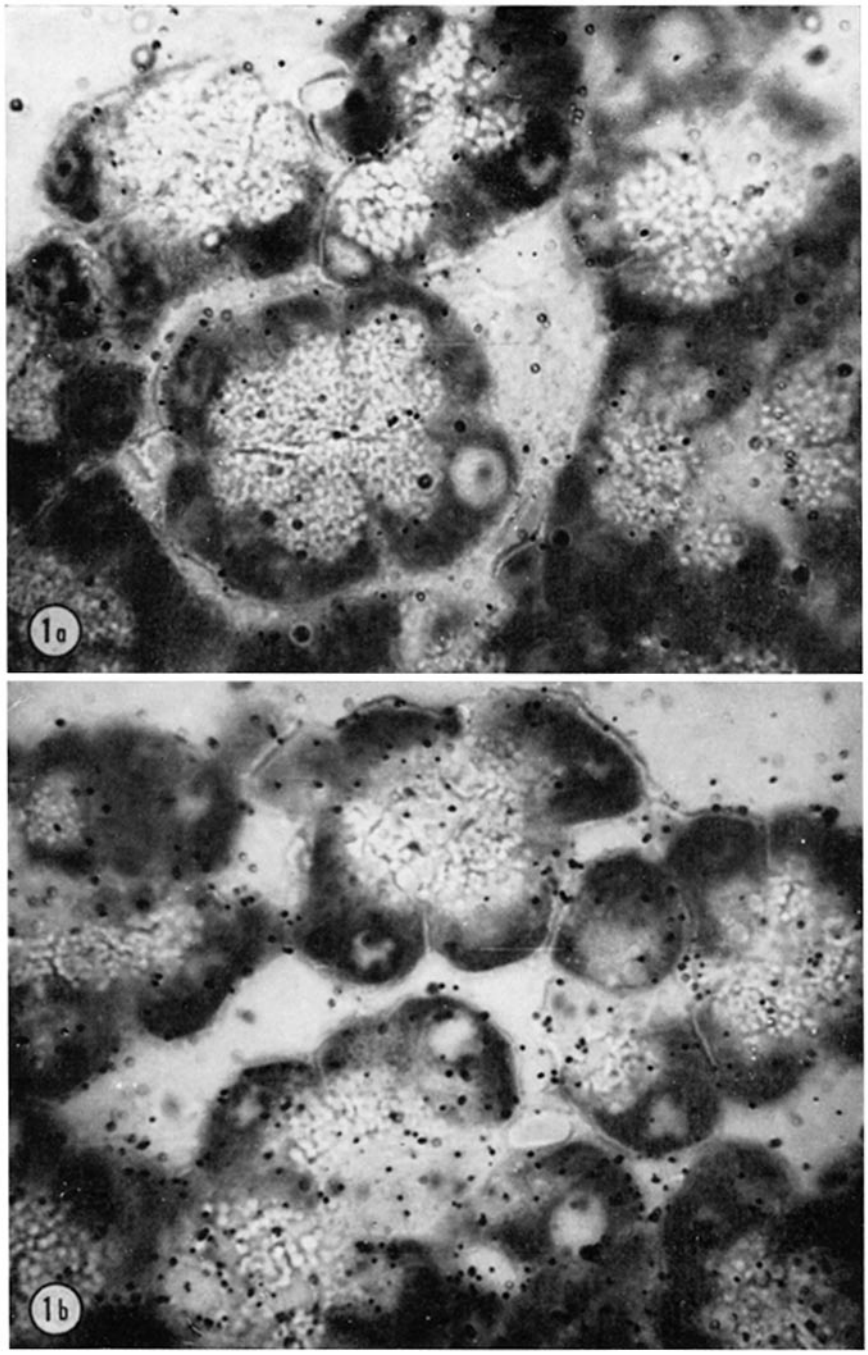

FIGURE 1 Radioautograms of guinea pig pancreas tissue incubated without and with pancreozymin in the presence of *myo*-inositol-2-^{3}H. *a*. Incubation without pancreozymin. *b*. Incubated with pancreozymin. Guinea pig pancreas slices were incubated for 30 min in 1.0 ml of Krebs-Henseleit bicarbonate saline containing 1.5 mg/ml glucose and 0.37 mM *myo*-inositol-2-^{3}H. The specific activity of the *myo*-inositol-2-^{3}H was 150 mc/mmole. After the initial 30-min incubation, 5 Ivy dogs units of pancreozymin were added to vessel b, and incubation was continued for another 30 min. Radioautograms of the tissue were then prepared as described under Materials and Methods. × 1100.

Grain Counting

Grain counts in whole fields were performed directly under the microscope and converted to grains per 100 μ^2 by suitable calibrations. Grain counting in various regions of the acinar cells was performed as follows. Photographs of a large number of fields were made under oil immersion. A penciled line was drawn on the photographic print between the basophilic cytoplasm, which stained dark with toluidine blue, and the nonbasophilic cytoplasm, which did not stain with toluidine blue. Usually the demarcation between these two regions was fairly sharp. Grains were then counted over nuclei, basophilic cytoplasm, and nonbasophilic cytoplasm. Although some grains might not be counted by this method because they would not be in focus, the percentage not in focus would be constant in all regions of the cell, so this method was regarded as valid for comparing different regions.

RESULTS

Radioautograms of Pancreatic Tissue Incubated without and with Pancreozymin in the Presence of myo-Inositol-2-^{3}H

Fig. 1 shows radioautograms of guinea pig pancreas slices incubated without and with pancreozymin in the presence of *myo*-inositol-2-^{3}H. The tissue incubated with pancreozymin showed considerably more grains over the acinar cells, as compared to the controls. Very few grains were located over the nuclei. Inspection of a large number of fields such as that shown in Fig. 1 failed to reveal a localization of grains to one part of the cytoplasm.

Grain Counts in Nuclei, Basophilic Cytoplasm, and Nonbasophilic Cytoplasm

In order to obtain a more quantitative picture of the distribution of grains in the cytoplasm, a large number of photographs were taken at random of different microscopic fields focused under oil immersion. A penciled line was drawn on the photographs separating the basophilic from the nonbasophilic cytoplasm. Grains were then counted over the nuclei, basophilic cytoplasm, and nonbasophilic cytoplasm, and the results are shown in Table I. In the unstimulated tissue, the grain density in the various cytoplasmic regions was not significantly above background (this was probably caused by the fact that, in this experiment, the background was counted over areas of slides without tissue and was subsequently found to be higher than over areas of slides containing nonradioactive tissue). However, in the pancreozymin-stimulated tissue the grain densities were significantly above background and significantly above the corresponding values in the unstimulated cells. There were significant increments in graining in both the nonbasophilic cytoplasm and the basophilic cytoplasm on stimulation with pan-

TABLE I

Effect of Pancreozymin on the Incorporation of myo-Inositol-2-^{3}H into Phosphatidylinositol in the Nonbasophilic and Basophilic Cytoplasm of the Pancreas Cell

Region	Grain density (Grains per 100 μ^2)			
	Unstimulated	Stimulated	Increment	*P*
Nonbasophilic cytoplasm	2.71 ± 0.08 (4)	7.97 ± 1.28 (4)	5.26	<0.01 > 0.001
Basophilic cytoplasm	3.24 ± 0.39 (6)	6.59 ± 0.78 (6)	3.35	<0.02 > 0.01
Nuclei	2.44 ± 0.54 (4)	3.62 ± 0.56 (4)	1.18	>0.1
Background	2.14 ± 0.71 (5)			

The grain counts were performed on slices from tissue taken from the experiment of Fig. 2. The background was determined over slides without tissue. The grain counts over the various areas are not corrected for background. The numbers in parentheses refer to the number of acini counted. The average area counted for nonbasophilic cytoplasm, basophilic cytoplasm, and nuclei from each acinus was 1016 μ^2, 1033 μ^2, and 284 μ^2. The average area for each background determination was 2514 μ^2.

creozymin. The increment in the nonbasophilic cytoplasm was generally somewhat greater than that in the basophilic cytoplasm.

Kinetics of Graining in Nonbasophilic and Basophilic Cytoplasm

There are two alternative explanations for the fact that graining increased in both the nonbasophilic cytoplasm and the basophilic cytoplasm. One is that the stimulation of *myo*-inositol-2-^{3}H incorporation into lipid occurs in one component—say the rough-surfaced endoplasmic reticulum, but that because of conversion of rough-surfaced membrane to smooth membrane the newly synthesized lipid ends up in smooth membrane. Alternatively, the increased synthesis of lipid may truly take place in both types of membrane. It was felt that kinetic studies of the graining in the two regions might throw light on this problem. If the first alternative were correct, one should observe a stimulation primarily in the basophilic region initially, followed by a gradually growing stimulation in the nonbasophilic region. If the second alternative were correct, one might expect to see parallel stimulations in both regions from the earliest times. Fig. 2 shows the incorporation of *myo*-inositol-2-^{3}H into the lipids of slices over a 30-min incubation period after the addition of ACh. The incorporation in the stimulated tissue was greater at the earliest sampling time (5 min) and continued more or less linearly until about 20 min after adding ACh. Fig. 3 shows the grain counts in nonbasophilic and basophilic cytoplasm from the same experiment. Stimulations were seen in both regions at the earliest sampling time, which was 2 min after adding ACh. The kinetic curves for grain counts were similar in shape to the kinetic curve for radioactivity in the lipids. The stimulations in both cytoplasmic regions were of the same order of magnitude, although, as in the experiment shown in Table I, the stimulation was greater in the nonbasophilic cytoplasm. It seems unlikely that a roughly equal stimulation in both regions at 2–5 min could have been due to conversion of rough membranes to smooth membranes, and vice versa. Although it is not proved, it seems a reasonable supposition, from the data, that *myo*-inositol-2-^{3}H incorporation was being stimulated independently in the basophilic region, which consists almost exclusively of rough-surfaced endoplasmic reticulum, and in the nonbasophilic region, which contains smooth-surfaced Golgi membranes.

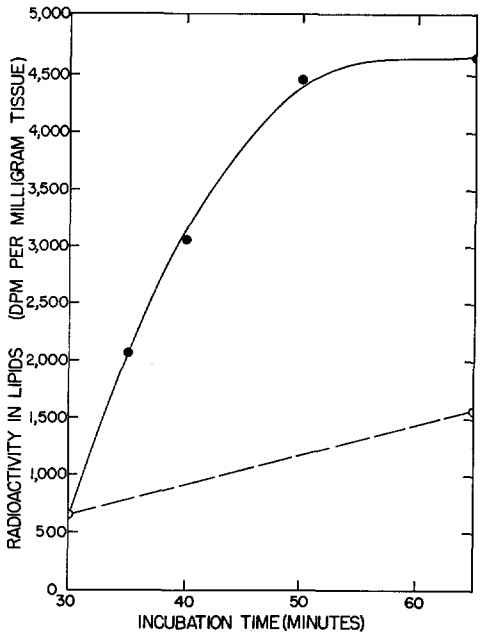

FIGURE 2 Incorporation of *myo*-inositol-2-^{3}H into the lipids of guinea pig pancreas slices incubated without and with acetylcholine. Guinea pig pancreas slices were incubated 30 min as described in Fig 1. 10^{-4} M acetylcholine plus 10^{-4} M eserine were then added to the vessels indicated by the solid circles. Slices were then removed at the indicated times and the total radioactivity in the lipids was determined as described under Materials and Methods.

Effect of Extraction of Tissue Sections with Chloroform-Methanol on Grain Counts

Table II shows the effect of extraction of tissue sections with 2:1 chloroform-methanol before radioautography. Almost all of the increment in graining due to incubation with pancreozymin or ACh + eserine was abolished if the sections were extracted with chloroform-methanol prior to radioautography. This suggests that the increment in grain counts on stimulation was due to incorporation of tritium into lipids. There was no significant reduction in graining in the unstimulated tissue. The explanation for this is not clear.

The grain density over areas of the slide without tissue was greater than over areas with nonradioactive tissue. This may be caused by a chemical suppression of graining by the tissue, to an absorp-

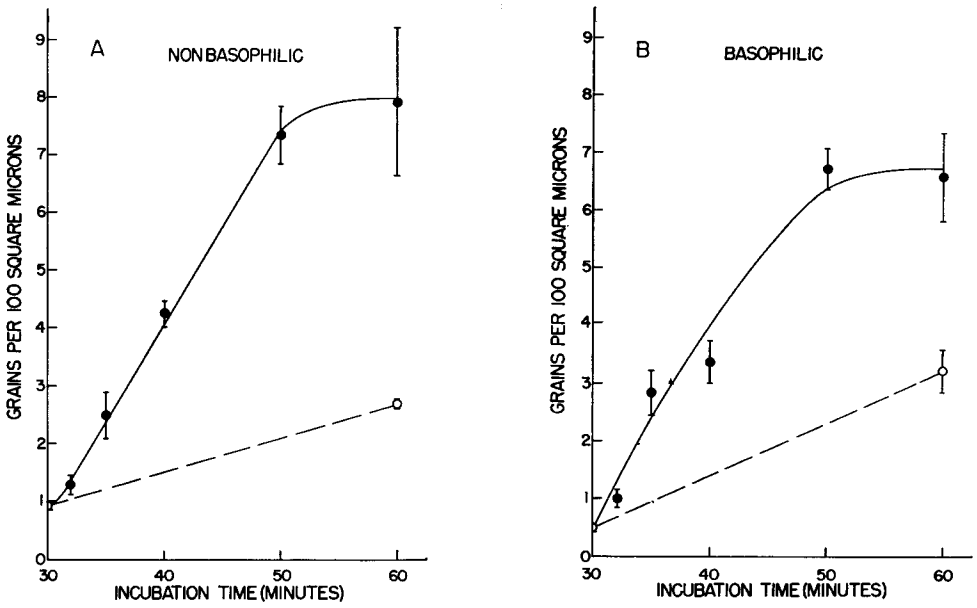

FIGURE 3 Kinetics of incorporation of *myo*-inositol-2-^{3}H into the lipids of nonbasophilic (3 *A*) and basophilic cytoplasm (3 *B*) in the presence and absence of acetylcholine. Open circles—controls. Solid circles—incubated with 10^{-4} M acetylcholine plus 10^{-4} M eserine. These grain counts were performed on tissue from the same experiment as in Fig. 2. The length of the vertical lines is twice the standard error of the mean. The grain counting was carried out as in Table I, except that 12 acini were counted for each point. The background was counted on each slide over an area without tissue. It ranged from 0.64 to 1.57 grains/100 μ^2. The values are corrected for background.

TABLE II

Effect of Extraction of Tissue Sections with Chloroform-Methanol on Grain Counts

Expt. No.	Additions	Net grain counts (Grains per 100 μ^2)	
		Unextracted	Extracted
1	None	2.46 ± 0.25 (10)	2.03 ± 0.53 (15)
	Pancreozymin	4.45 ± 0.49 (20)	2.44 ± 0.42 (30)
2	None	0.32 ± 0.31 (20)	0.92 ± 0.38 (20)
	ACh + eserine	4.38 ± 0.45 (30)	1.74 ± 0.25 (30)

The values are the mean, ±SEM, and the number of fields examined. Each field was 550 μ^2. The net grain counts are differences between grain counts over the tissue and over the glass from the same slide. The average net grain counts for nonradioactive tissue in experiments 1 and 2 were −0.62 and −1.27, respectively.

tion of low energy radiation from the glass, or to chemical graining due to the glass.

Further evidence that the stimulated incorporation of *myo*-inositol-2-^{3}H was into lipid was shown by an experiment in which the average ratio of grain counts in the stimulated tissue to that in the unstimulated tissue was compared with the ratio of the radioactivities in the lipid extract from the corresponding tissues. After correction for background, the ratio of grain densities was 3.7; the corresponding ratio for radioactivities in the lipid extracts was 3.8.

Demonstration that the Stimulated Incorporation of myo-Inositol-2-^{3}H Is into Phosphatidylinositol

In a previous publication (1), it was shown that essentially all of the radioactivity recovered in the washed chloroform-ethanol extracts of trichloroacetic acid–treated pigeon pancreas tissue previously incubated with *myo*-inositol-2-^{3}H could be accounted for by incorporation into phosphatidylinositol. Fig. 4 shows radioactive scans of chromatograms of total lipid extracts of guinea pig pancreas slices incubated without and with pancreozymin in the presence of *myo*-inositol-2-^{3}H. This chromatographic system clearly separates phosphatidylinositol, diphosphoinositide, and triphosphoinositide (9). There were radioactive peaks in the areas occupied by standard phosphatidylinositol spots. The peak from the unstimulated tissue was at the limit of detection. This is caused by the fact that the counting efficiency for tritium on chromatographic paper was 0.1%. A maximum amount of lipid (from about 10 mg of tissue) was run on each chromatogram—approximately 18,000 dpm in the unstimulated slices, and approximately 60,000 dpm in the stimulated slice. The important points in Fig. 4 are that the radioactive peak from the tissue stimulated with pancreozymin coincided with that of standard phosphatidylinositol and that there was no evidence of radioactivity in the polyphosphoinositide regions of the chromatogram. These results confirm and extend the earlier studies with pigeon pancreas (1). They indicate that essentially all of the increase in radioactivity in the total lipid extract on stimulation of secretion is due to increased incorporation of tritium into phosphatidylinositol and that the polyphosphoinositides do not contribute significantly to the total lipid radioactivity under these conditions.

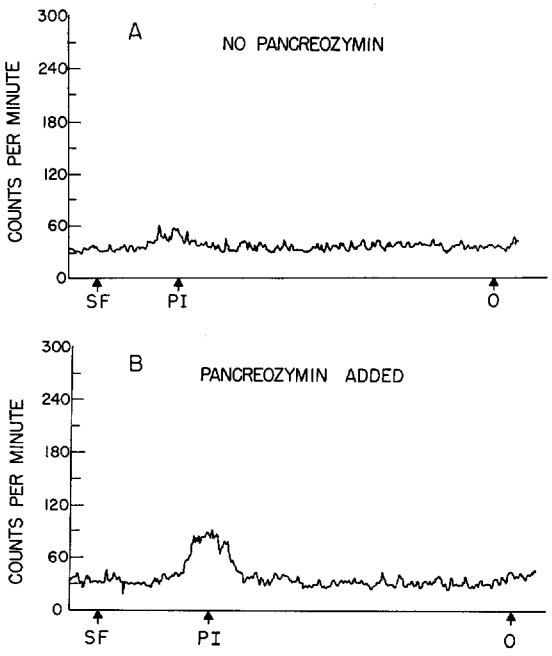

FIGURE 4 Radioactive scan of chromatograms of total lipid extracts of guinea pig pancreas slices incubated without (4 *A*) and with (4 *B*) pancreozymin. *O* = origin; *PI* = phosphatidylinositol; *SF* = solvent front. The R_f of phosphatidylinositol varies somewhat in this chromatographic system.

DISCUSSION

The Site of the Phosphoinositide Effect Associated with Stimulation of Protein Secretion

The salient observation in this paper is that the increased incorporation of *myo*-inositol-2-^{3}H into phosphatidylinositol, previously shown to be in microsomal components (2), occurred in the basophilic and nonbasophilic cytoplasm. Recently, Jamieson and Palade (3) have shown that the microsome fraction from guinea pig pancreas contains rough and smooth membranes derived from the endoplasmic reticulum and the Golgi membranes, respectively. Since the smooth and rough membranes occupy rather characteristic regions in the pancreatic acinar cell, the combined data from earlier differential centrifugation studies of Redman and Hokin (2) and the present radioautographic studies allow us to determine in which membrane type or types the phosphoinositide effect occurs. If the phosphoinositide effect were exclusively in rough membranes, the increase in graining would be primarily in the basophilic cytoplasm, since the concentration of rough-surfaced membranes is very much greater in this region than in the nonbasophilic cytoplasm. If the phosphoinositide effect were in smooth membranes only, the increased graining would be almost exclusively in the nonbasophilic cytoplasm, since there are very few smooth membranes in the basophilic cytoplasm. Since there were independent increases in graining in both basophilic cytoplasm and nonbasophilic cytoplasm, it can be concluded that the increased incorporation of *myo-*

inositol-2-^{3}H into phosphatidylinositol was in both rough and smooth membranes. Kinetic studies of the ACh-stimulated incorporation in these two regions provide no evidence that the stimulation is primarily in one region only and that the stimulation in the other is due to conversion of one type of membrane to the other.

Role of Phosphatidylinositol in Membrane Circulation Associated with Protein Secretion

In attempting to assign a role of phosphatidylinositol turnover to the over-all secretory process, it is necessary to consider membrane circulation in the pancreas. Resulting largely from the efforts of Palade and his associates (11), the following picture of membrane circulation in the pancreas has emerged. The secretory protein which is newly synthesized is transported across the membranes of the rough-surfaced endoplasmic reticulum into the cisternal spaces in which it accumulates (Redman et al., 12). By a mechanism which is as yet unclear, the secretory protein from the cisternal spaces is transferred to small smooth-surfaced vesicles containing secretory protein (see Jamieson and Palade, 3). This may involve budding of smooth-surfaced membrane from the endoplasmic reticulum in the Golgi region. By coalescence of the small vesicles and possibly by addition of membrane from the Golgi bodies, the membranes of the mature zymogen granules are formed. Protein is concentrated in the zymogen granules by extrusion of water and possibly by transport of additional protein across the zymogen granule membranes (see Hokin, 13, for a discussion of the latter point). When enzyme extrusion is triggered, the membranes of the zymogen granules coalesce with the plasmalemma, the membranes part at the point of fusion, and the secretory proteins are discharged into the lumen. In this scheme, it must be assumed that membrane which is added to plasmalemma is returned somehow to the intracellular membrane system.

A detailed discussion of the possible significance of the data on phospholipid turnover in relation to protein secretion in the pancreas is given elsewhere (13, 14). Fig. 5 shows one way of explaining the data. According to this scheme, when the pancreatic acinar cell is in contact with the secretogogue, the contents of the zymogen granules are discharged by "reverse pinocytosis" at the plasmalemma and, concomitantly, new intracellular membrane is formed. The membrane which is added to the plasmalemma by coalescence with zymogen granules breaks down into its constituents and these constituents are reassembled in new smooth and rough intracellular membranes, replacing that which was lost to the zymogen granules. In the process of membrane breakdown, phosphatidylinositol is degraded to its building blocks—glycerol, phosphate, fatty acids, and inositol—but lecithin is not. This can account for the fact that on stimulation of secretion there is increased incorporation of glycerol, phosphate, and inositol into phosphatidylinositol, but no increased turnover of lecithin (1). It is possible that lecithin is contained in subunits which contain protein and other phospholipids as well and that the individual phospholipids in the subunits do not break down to their building blocks during membrane circula-

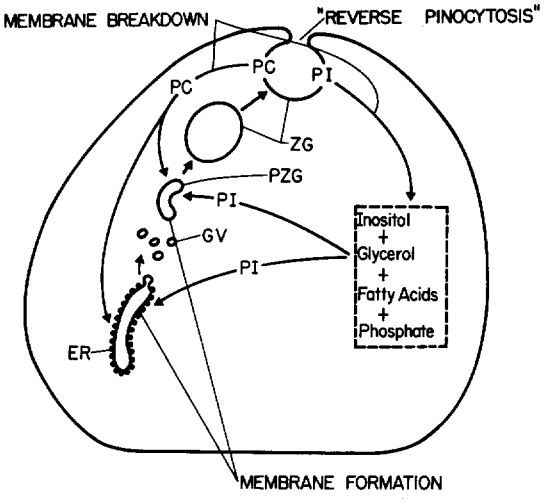

FIGURE 5 Possible interpretation of role of phosphatidylinositol turnover in protein secretion in pancreas. When protein secretion is stimulated, the membrane of the zymogen granule coalesces with the plasmalemma and discharges its contents by "reverse pinocytosis." The zymogen granule membrane then breaks down, releasing the molecular constituents of phosphatidylinositol and undegraded phosphatidylcholine. Phosphatidylinositol is then resynthesized in the endoplasmic reticulum and in the Golgi membranes to replace that membrane which contributed to the formation of the zymogen granule membrane. Phosphatidylcholine is reassembled in the endoplasmic reticulum and in the Golgi membranes without undergoing breakdown and resynthesis. Abbreviations: *ZG*, zymogen granules; *PZG*, prozymogen granules; *GV*, Golgi vesicles; *ER*, endoplasmic reticulum; *PI*, phosphatidylinositol; *PC*, phosphatidylcholine.

tion. It has been suggested (13, 14) that phosphatidylinositol might act as a "cement" between subunits and that its breakdown and resynthesis controls the relocation of the subunits from the plasmalemma to the intracellular membranes. However, there is no direct evidence concerning the existence of subunits in pancreatic acinar membranes, so this interpretation must remain speculative at this time.

Kinetic studies and studies with different radioactive precursors indicate that the phospholipid effects in the pancreatic acinar cell are different from those occurring in the salt gland on stimulation of NaCl secretion with acetylcholine (15). Detailed studies of the phospholipid effects in other protein-secreting glands have not been carried out, but it seems likely that the phospholipid effects observed in these other glands (16) are related to those in the pancreas. Arguments have been put forward that the phospholipid effects observed in synaptic tissue on exposure to acetylcholine resemble more closely those in the pancreas in that they appear to be related to intracellular membrane synthesis associated with vesicle formation (17).

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