Characterization of Phospholipids in Insulin Secretory Granules and Mitochondria in Pancreatic Beta Cells and Their Changes with Glucose Stimulation*

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Michael J. MacDonald‡§, Lacmboue Adé, James M. Ntambi¶‡, Israr-Ul H. Ansari‡, and Scott W. Stoker‡

From the ‡Children’s Diabetes Center, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin 53706 and the Departments of §Biochemistry and ¶Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706

Background: Phospholipids in insulin granules were characterized.

Results: Phosphatidylserine and phospholipidswith unsaturated or short fatty acids were concentrated in granules and increased with glucose stimulation.

Conclusion: Phosphatidylserine enhances the interaction between proteins in granules and plasma membranes. Unsaturated and short FA increase the fluidity and curvature of lipid bilayers.

Significance: Fusion of granules to PM and insulin exocytosis is enhanced.

The lipid composition of insulin secretory granules (ISG) has never previously been thoroughly characterized. We characterized the phospholipid composition of ISG and mitochondria in pancreatic beta cells without and with glucose stimulation. The phospholipid/protein ratios of most phospholipids containing unsaturated fatty acids were higher in ISG than in whole cells and in mitochondria. The concentrations of negatively charged phospholipids, phosphatidylinerine, and phosphatidylinositol in ISG were 5-fold higher than in the whole cell. In ISG phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine, and sphingomyelin, fatty acids 12:0 and 14:0 were high, as were phosphatidylserine and phosphatidylinositol containing 18-carbon unsaturated FA. With glucose stimulation, the concentration of many ISG phosphatidylserines and phosphatidylinositols increased; unsaturated fatty acids in phosphatidylserine increased; and most phosphatidylethanolamines, phosphatidylcholines, sphingomyelins, and lysosphatidylcholines were unchanged. Unsaturation and shorter fatty acid length in phospholipids facilitate curvature and fluidity of membranes, which favors fusion of membranes. Recent evidence suggests that negatively charged phospholipids, such as phosphatidylserine, act as coupling factors enhancing the interaction of positively charged regions in SNARE proteins in synaptic or secretory vesicle membrane lipid bilayers with positively charged regions in SNARE proteins in the plasma membrane lipid bilayer to facilitate docking of vesicles to the plasma membrane during exocytosis. The results indicate that ISG phospholipids are in a dynamic state and are consistent with the idea that changes in ISG phospholipids facilitate fusion of ISG with the plasma membrane-enhancing glucose-stimulated insulin exocytosis.

Glucose and other metabolizable insulin secretagogues stimulate insulin secretion not only by providing energy but also via mitochondrial biosynthesis. Pancreatic beta cell mitochondria have an enormous capacity for synthesis of molecules from insulin secretagogues and the export of their metabolites to the extramitochondrial compartment of the cell. This includes short chain acyl-CoAs, the precursors of lipids (1–10), and it so happens that the beta cell is a very lipogenic tissue (9, 11–16). One widely recognized pathway of synthesis of lipid precursors in the beta cell is anaplerosis (17) that involves the mitochondrial net synthesis and replenishment of citric acid cycle intermediates combined with cataplerosis, the export of these intermediates to the extramitochondrial space of the cell (1–4, 7–9, 18–24). There also appears to be another pathway of mitochondrial biosynthesis in the beta cell. This pathway uses the export of acetoacetate synthesized in mitochondria and its export to the cytosol (5, 6, 21, 25–29). Both the acetoacetate pathway and the anaplerotic/cataplerotic pathway can provide short chain acyl-CoAs from glucose as precursors for the synthesis of cellular lipids (Fig. 1). The anaplerosis pathway involves the synthesis of citrate from oxaloacetate formed by the anaplerotic enzyme pyruvate carboxylase in mitochondria and citrate’s export to the cytosol, where it is converted to acetyl-CoA, a precursor of lipid synthesis, catalyzed by ATP citrate lyase (1–4, 8, 9, 19, 22–24). Indeed, knockdown of pyruvate carboxylase in the beta cell with siRNA technology inhibits glucose-induced lipid synthesis (12) and insulin release (23, 24). The acetoacetate pathway involves the synthesis of acetoacetate via succinyl-CoA:3-ketoacid-CoA transferase in mitochondria and the export of acetoacetate to the cytosol, where it is converted to acetoacetyl-CoA by acetoacetyl-CoA synthetase and then to other short chain acyl-CoAs that are precursors for lipid synthesis (Fig. 1). We have proposed that a major purpose of mitochondrial biosynthesis in the beta cell is to provide precursors for lipid synthesis (4, 5, 11, 12, 22, 23, 29). Interestingly, for short chain acyl-CoA biosynthesis, human pancreatic islets use succinyl-CoA:3-ketoacid-CoA transferase plus acetoacetyl-CoA synthetase more than anaplerosis/cataplerosis via
pyruvate carboxylase plus ATP citrate lyase compared with rodent islets or rodent insulin cell lines (29), which have been the most common models used for many decades to establish ideas about beta cell metabolism.

Although the rate of lipogenesis in the beta cell can be very high, it is tightly regulated. Too little synthesis of lipids is associated with lower insulin release (7, 11, 12, 14, 30), and excess lipid in the beta cell derived from excessive lipid synthesis or lipids from exogenous sources is harmful to the beta cell (7, 13–16). The first step in lipid synthesis in the cytosol involves the conversion of acetyl-CoA into malonyl-CoA catalyzed by acetyl-CoA carboxylase (ACC).2 Then fatty acid synthase (FAS) adds malonyl-CoA carbon as C2 units to a growing fatty acid chain. Rat and human pancreatic islets and rat insulinoma cell lines contain a high concentration of ACC, and the isoform of ACC that these cells contain is ACC1, which is the isoform found in very lipogenic tissues, such as liver and adipose tissue (11). Although the rat insulinoma INS-1 832/13 cell line (11) and human pancreatic islets (11, 29) contain a high level of FAS, rat pancreatic islets contain only a small amount of this enzyme (29, 31).

We previously observed that phosphatidylinerine (PS) and several other (phospho)lipids increase 10–20% in pancreatic beta cells during glucose stimulation over a time course coincident with insulin secretion (11). Also, small molecule inhibitors of ACC and FAS inhibit glucose- or BCH (2-amino-2-methyl-1,3-propanediol) plus glutamine-stimulated insulin release from rat pancreatic islets and INS-1 832/13 cells (11). By using shRNA technology, we recently generated multiple INS-1 832/13-derived cell lines with knocked down FAS or PC. Glucose-stimulated synthesis of certain (phospho)lipids and insulin release was strongly inhibited in these cell lines (12). (There is, however, one report of FAS knockdown with a single virus-delivered siRNA failing to inhibit insulin release in these cells (32)). The above results suggest that (phospho)lipids are some of the products of acute biosynthesis in the beta cell and that alterations in the cellular lipid composition are necessary for insulin secretion.

It would seem from a priori reasoning that the lipids in insulin secretory granule (ISG) membranes need to undergo enormous remodeling during insulin exocytosis. Available evidence suggests that metabolism is necessary for both the first and second phase of insulin secretion and establishes full release competence of ISG (33, 34). Rapid remodeling of lipid in intracellular membranes seems especially necessary for insulin exocytosis during proinsulin synthesis in the endoplasmic reticulum and as proinsulin traverses the cis-Golgi network and is packaged into nascent granules at the trans-Golgi, and the ISG membranes are modified as ISG continue to mature until the ISG lipid bilayer fuses with the plasma membrane lipid bilayer, where insulin is finally extruded into the circulation. Phospholipids (PLs) are the major lipids in cellular membranes. Therefore, it was reasonable to determine whether the phospholipid composition of the ISG changes during exocytosis.

Although ISG proteins, especially SNARE proteins, have been studied in numerous excellent laboratories, there has never been even one report of a comprehensive characterization of lipids in ISG similar to reports of granules or vesicles from other tissues. In this report, we describe an extensive characterization of phospholipids in ISG in pure beta cells (INS-1 832/13 cells) and changes in their composition with glucose stimulation over a time course coincident with the early stages of sustained metabolism-stimulated insulin secretion. In addition, we compared the same lipids in ISG with their concentrations in whole cells and mitochondria. We did not study cholesterol because there have been studies of cholesterol in ISG (16, 30, 35). The results indicate that negatively charged PLs,
including PS and PL, increase in ISG during glucose stimulation. Shorter FA length in PL and unsaturated FA in PL favor membrane curvature and membrane fluidity that would enhance fusion and docking of the ISG bilayer with the plasma membrane bilayer. Shorter chain FA in PL and unsaturation in PL FA increased with glucose stimulation.

Flipases are P4 ATPases that catalyze translocation of PS and PE from the luminal side to the cytosolic side of a secretory or synaptic vesicle lipid bilayer and from the extracellular side to the cytosolic side of the plasma membrane lipid bilayer. The negatively charged PS interacts with regions of basic amino acids in SNARE proteins in the vesicle and with regions of basic amino acids in SNARE proteins in the plasma membrane to couple and enhance docking and fusion of the two membranes, thus facilitating exocytosis of proteins. The flipases that are present in beta cells, including in the ISG, have been identified. In line with the idea that phospholipids are in a dynamic state in ISG and that changes in PLS facilitate the interaction of the ISG membrane with the plasma membrane during insulin exocytosis, knockdown of the flipases identified in INS-1 832/13 beta cells or subcellular fractions was measured as described under “Experimental Procedures.” Results are the means ± S.E. of four experiments.

**EXPERIMENTAL PROCEDURES**

**Materials**—[U-14C]glucose was from PerkinElmer Life Sciences. Silica gel 60 thin layer chromatography plates were from EMD Millipore. INS-1 832/13 cells were from Christopher Newgard (36). All other chemicals were from Sigma-Aldrich in the highest purity available.

**Incubation of Cells to Measure [14C]Glucose Incorporation**—INS-1 832/13 cells were maintained as monolayers on 150-mm tissue culture plates in the presence of RPMI medium (contains 11.1 mM glucose), 10% FCS, 1 mM pyruvate, and 50 μM β-mercaptoethanol (INS-1 medium) until cells were 80–90% confluent. Cells were then maintained in INS-1 medium modified to contain 5 mM glucose (Table 1 experiment) (3 mM glucose for the Fig. 2 experiment) and no pyruvate or β-mercaptoethanol for 20 h followed by incubation for 3 h in RPMI 1640 medium containing 1 mM glucose. To measure glucose incorporation into subcellular fractions, cells were incubated for 30 min (Table 1 experiment) or 45 min (Fig. 2 experiment) in Krebs Ringer bicarbonate buffer solution (modified to contain 15 mM sodium HEPES buffer and 15 mM lower NaCl), pH 7.3, containing 1 mM or 10 mM [U-14C]glucose (specific radioactivity, 0.4 mCi/mmol glucose (Table 1)), harvested, and fractionated into subcellular fractions using differential centrifugation as described below. Subcellular fractions were then washed 6–15 times with trichloroacetic acid until the radioactivity in the supernatant fractions of the washed material equaled background levels (Table 1). In separate experiments, the total lipid fraction was extracted directly without acid-washing and saponified to measure glucose incorporation into glyceride fatty acid and glyceride glycerol fractions in INS-1 832/13 cells (Fig. 2). This was done as described previously for experiments with adipocytes (37) except that one-fifth the volume of all reagents for lipid extractions was used.

**Subcellular Fractionation of INS-1 832/13 Cells**—Monolayers of INS-1 832/13 cells maintained on 150-mm tissue culture plates as described below were placed on ice and quickly washed twice with cold phosphate-buffered saline. Cells were scraped from the plates into a 1.5-ml microcentrifuge test tube and centrifuged at 600 × g for 20 s. All subsequent steps were at 4°C. Subcellular fractions were prepared with slight modifications of our (38–40) and others’ (41–44) previously described methods. The cell pellet was suspended in 1 ml of 220 mM mannitol, 70 mM sucrose, 5 mM potassium HEPES buffer, pH 7.5 (KMSH) containing 0.5 mM EGTA and 0.5 mM EDTA (per one or two 150-mm plates) and homogenized with 40 strokes up and down in a Potter-Elvehjem homogenizer. The method was designed to obtain pure ISG free from mitochondria. This resulted in slight contamination of mitochondria with ISG. The homogenate was centrifuged at 600 × g for 10 min, and the resulting supernatant fraction was centrifuged at 12,000 × g for 10 min to generate a mitochondrial pellet. The supernatant fraction from this centrifugation was centrifuged at 20,800 × g for 20 min to generate the insulin granule pellet. This pellet was washed up to three times with KMSH at 20,800 × g for 20 min and used for lipid analysis. The resulting ISG were ≈90% pure based on immunoblotting of VAMP2 to detect ISG and the absence of mitochondrial glycerol phosphate dehydrogenase enzyme activity, a marker for beta cell mitochondria in the ISG (data not shown).

**Lipid Analyses**—INS-1 832/13 cells at 90% confluence were maintained on 150-mm tissue culture plates in INS-1 tissue culture medium containing 3 mM glucose and no pyruvate or β-mercaptoethanol for 20 h prior to an experiment. Plates were washed twice with warm phosphate-buffered saline, and cells were incubated in INS-1 medium (RPMI 1640 medium containing 10% FCS) without pyruvate or β-mercaptoethanol containing either no glucose or 16.7 mM glucose. After 2 h at 37°C, cells were scraped from each plate into individual test tubes, and 50 μl of the suspension was removed and saved for estimation of total protein. Lipids were analyzed in whole cells or subcellular fractions. Chloroform/methanol (2:1) containing 0.01% butylated hydroxytoluene (4 ml/test tube) was added, cells or subcellular fractions were vortexed vigorously for 30 min, and the mixture was allowed to set for 22 h in capped

### Table 1

**Increased incorporation of radioactivity into subcellular fractions of pancreatic cells stimulated with 10 mM [U-14C]glucose versus 1 mM [U-14C]glucose**

| Subcellular fraction                  | Percentage of homogenate ± S.E. | -Fold 10 mM glucose (%) |
|--------------------------------------|---------------------------------|------------------------|
| Homogenate                           | 100 ± 3.5                       | 8.9 ± 0.5              |
| Insulin secretory granule            | 20 ± 2.4                        | 8.5 ± 1.7              |
| Mitochondria                         | 22 ± 1.5                        | 17.4 ± 0.7             |
| Plasma membrane                      | 13 ± 0.9                        | 10.9 ± 1.3             |
| Cytosol                              | 10 ± 1                          | 11.4 ± 1.1             |
| Nuclei and cell debris               | 26 ± 5                          | 5.9 ± 0.5              |

In INS-1 832/13 cells were incubated for 30 min with high or low glucose, and glucose incorporation into various subcellular fractions was measured as described under “Experimental Procedures.” Results are the means ± S.E. of four experiments.

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methylated by boiling in 14% boron trifluoride in hydroxytoluene. The extract was evaporated to dryness, and into chloroform/methanol (4:1) containing 0.01% butylated hydroxytoluene. Lipid classes were scraped from the plates and extracted (45) and separated on Silica gel 60 thin layer chromatography tubes. Lipids were extracted by the method of Bligh and Dyer (45) and separated on Silica gel 60 thin layer chromatography plates. Lipid classes were scraped from the plates and extracted into chloroform/methanol (4:1) containing 0.01% butylated hydroxytoluene. The extract was evaporated to dryness, and lipids were methylated by boiling in 14% boron trifluoride in methanol containing 20 or 2 nmol of pentadecanoic acid as a standard. Hexane and water were added, and the mixture was centrifuged. The organic layer was transferred to a test tube and evaporated under nitrogen and resuspended in hexane and analyzed by gas chromatography, as described previously (11, 12, 46) except that 1.5 ml instead of 4 ml of chloroform/methanol/butylated hydroxytoluene was added to each test tube for lipid extraction.

**Data Analysis**—Statistical significance, when calculated, was confirmed with Student’s t test. Statistical analysis on the measurements of individual lipids is not shown in Figs. 3–14. Measured small differences with glucose stimulation versus without glucose stimulation among levels of individual lipids present at high concentrations could be biologically relevant whether statistically significant or not. In addition, because of the intrinsic low accuracy of measurements of the lipids present at very low concentrations, observed large relative differences among individual lipids present at very low concentrations, although statistically significant, may or may not be real. The general consistency in the directions of the changes in many of the lipids and the positive (or negative) correlation with glucose-stimulated insulin release suggests that the individual and collective changes have biological meaning regardless of the statistical significance of the changes.

**RESULTS**

**Preparation of Insulin Granules and Mitochondrial Fractions**—The emphasis of the current work was on insulin granules. Mitochondria were studied as a conveniently obtained organelle for comparison with insulin granules and were interesting in themselves and because they are the site of metabolism and formation of several PLs, such as in the conversion of PC and PE to PS. Mitochondrial contamination of ISG has been a major problem with purification schemes used to obtain ISG. The scheme for preparation of ISG for lipid analysis was designed to obtain pure granules, by sacrificing the yield of ISG in order to obtain more pure ISG completely free of mitochondria. This resulted in a slight contamination of mitochondria with ISG. ISG used for lipid analysis were washed three times and were 90% pure. An immunoblot with an antibody against VAMP2, a marker for ISG, showed that the ISG fraction used contains a high concentration of this marker and no mitochondrial glycerol phosphate dehydrogenase enzyme activity in the ISG fraction (data not shown). Mitochondrial glycerol phosphate dehydrogenase is present only in mitochondria against VAMP2, a marker for ISG, showed that the ISG fraction used contains a high concentration of this marker and no mitochondrial glycerol phosphate dehydrogenase enzyme activity in the ISG fraction (data not shown). Mitochondrial glycerol phosphate dehydrogenase is present only in mitochondrial mitochondria.
| Fatty Acid | ISG Phosphatidylserine | Mito Phosphatidylserine | Whole cell Phosphatidylserine | ISG Phosphoinositol | Mito Phosphoinositol | Whole cell Phosphoinositol | ISG Phosphoethanolamine | Mito Phosphoethanolamine | Whole cell Phosphoethanolamine | ISG Lysophosphatidylcholine | Mito Lysophosphatidylcholine | Whole cell Lysophosphatidylcholine | ISG Sphingomyelin | Mito Sphingomyelin | Whole cell Sphingomyelin | ISG Phosphatidylcholine | Mito Phosphatidylcholine | Whole cell Phosphatidylcholine |
|------------|------------------------|------------------------|-------------------------------|----------------------|----------------------|----------------------------|--------------------------|--------------------------|-----------------------------|-----------------------------|-----------------------------|----------------------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 12:0       | 3.0                    | 2.2                    | 1.3                           | 4.7                  | 9.9                  | 0.5                        | 1.5                      | 0.04                     | 0.4                         | 1.5                      | 2.1                      | 0.7                        | 12                        | 11                      | 1.9                        | 16                      | 15                      | 4.5                        |
| 14:0       | 9.4                    | 7.2                    | 2.1                           | 5.1                  | 11                   | 1.3                        | 3.0                      | 1.3                      | 0.4                         | 9.1                       | 7.2                      | 6.9                        | 16                        | 15                      | 4.5                        | 8.0                      | 6.6                      | 3.7                        |
| 14:1       | 0.6                    | 0.1                    | 0.1                           | 0.1                  | 0.1                  | 0.1                        | 0.3                      | 0.3                      | 0.1                         | 0.3                       | 0.1                      | 0.1                        | 0.1                      | 0.1                      | 0.01                       | 0.1                      | 0.1                      | 0.01                       |
| 16:0       | 29                    | 26                    | 18                            | 29                   | 18                   | 22                        | 39                      | 20                      | 13                         | 27                       | 46                      | 42                        | 31                        | 37                      | 33                        | 54                       | 53                      | 35                        |
| 16:1 (n-10)| 1.6                   | 2.7                   | 5.5                           | 2.5                  | 2.6                  | 3.9                        | 0.8                      | 1.4                      | 2.6                         | 2.1                       | 16                      | 20                        | 18                       | 16                      | 21                        | 14                       | 12                      | 7.8                        |
| 18:0       | 0.1                   | 0.3                   | 0.3                           | 0.1                  | 0.1                  | 0.1                        | 0.3                      | 0.3                      | 0.7                         | 0.4                       | 0.7                      | 0.5                        | 0.4                       | 0.3                      | 1.2                        | 1.2                       | 1.1                      | 0.2                        |
| 18:1 (n-9) | 0.6                   | 0.5                   | 0.2                           | 0.2                  | 0.2                  | 0.2                        | 0.2                      | 0.2                      | 0.2                         | 0.2                       | 0.2                      | 0.2                        | 0.2                       | 0.2                      | 0.2                        | 0.2                       | 0.2                      | 0.2                        |
| 18:2 (n-6) | 0.4                   | 0.3                   | 0.3                           | 0.3                  | 0.3                  | 0.3                        | 0.3                      | 0.3                      | 0.3                         | 0.3                       | 0.3                      | 0.3                        | 0.3                       | 0.3                      | 0.3                        | 0.3                       | 0.3                      | 0.3                        |
| 20:0       | 30                    | 43                    | 24                            | 38                   | 26                   | 30                         | 15                      | 27                      | 39                         | 41                       | 29                      | 27                        | 32                       | 28                      | 29                        | 23                       | 28                      | 52                        |
| Total      | 100                   | 100                   | 100                           | 100                  | 100                  | 100                         | 100                      | 100                     | 100                         | 100                      | 100                     | 100                        | 100                      | 100                     | 100                        | 100                      | 100                     | 100                        |
Insulin Granule Phospholipids in Pancreatic Beta Cells

Glucose Incorporation into Insulin Granules and Mitochondria—With respect to insulin release, the glucose response curve of INS-1 832/13 cells is left-shifted compared with pancreatic islets, and nearly maximal insulin release in these cells is seen at 11 mM glucose (36). INS-1 832/13 cells were incubated with $^{14}$C-labeled 1 mM glucose and 10 mM glucose for 30 min. In the presence of 10 mM glucose, the amount of glucose carbon incorporated into ISG and mitochondria was 20 and 22%, respectively, of the total glucose carbon incorporated into the whole cell. The amount of glucose carbon incorporated into the ISG and mitochondria was 8 and 4 nmol/mg of protein, respectively. This represented an 8.5-fold increase in the incorporation of label into the ISG compared with in the presence of 1 mM glucose (Table 1). Fig. 2 shows glucose incorporation into glyceride fatty acid and glyceride glycerol moieties of phospholipids and triglycerides in INS-1 832/13 cells incubated for 45 min in the presence of 10 or 1 mM glucose. As expected, most of the incorporation of glucose was into the glycerol backbone of PL and triglycerides. However, the glucose incorporated into glyceride fatty acids was 4.1-fold higher in ISG and 4.8-fold higher in mitochondria in the presence of 10 mM glucose compared with 1 mM glucose (Fig. 2), indicating that rapid de novo synthesis of fatty acids in PL and triglycerides can occur in the beta cell.

PL Composition of Insulin Granules—Table 2 shows the percentages of various PL in ISG compared with values from the literature for adrenal chromaffin granules, brain synaptic vesicles, and pancreas acinar granules (48–54). The PL classes of ISG most closely resemble that of bovine, rat, and human adrenal gland chromaffin granules. The most striking difference between chromaffin granules and ISG was the exceptionally high percentage of PI of 21% in ISG (versus 2% in chromaffin granules), which was most similar to the high level of PI in pancreatic granules (Table 2). The percentage of PS of 10% in ISG was about the same as in chromaffin granules and brain synaptic vesicles. The percentage of PE in ISG was about 70% of that in chromaffin granules, and PC was about 60% of the percentages in chromaffin and pancreatic granules and brain synaptic vesicles (Table 2). The percentage of sphingomyelin (SM) of 10% in ISG was about the same or slightly lower than the percentage of SM in chromaffin granules and pancreatic granules.
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**Phosphatidylserine**

| Fatty Acid | Unstimulated Mitochondria (nmol/mg protein) | Unstimulated Whole Cell (nmol/mg protein) | Mitochondria to Whole Cell Ratio | Percent Change vs Unstimulated Control Mitochondria |
|------------|---------------------------------------------|------------------------------------------|----------------------------------|-----------------------------------------------------|
| 12:0       | 0.4 ± 0.04                                  | 0.1 ± 0.02                               | 4.0                              | NC                                                  |
| 12:1       | ND                                          | 0.03 ± 0.03                              | NC                               | NC                                                  |
| 14:0       | 1.3 ± 0.3                                   | 0.1 ± 0.01                               | 13                               | NC                                                  |
| 14:1       | ND                                          | 0.01 ± 0.01                              | NC                               | NC                                                  |
| 16:0       | 4.9 ± 1.7                                   | 0.8 ± 0.01                               | 6.1                              | NC                                                  |
| 16:1       | 0.5 ± 0.2                                   | 0.3 ± 0.1                                | 1.7                              | NC                                                  |
| 18:0       | 2.7 ± 0.4                                   | 2.4 ± 0.5                                | 1.1                              | NC                                                  |
| 18:1(n-9)  | 4.5 ± 2.4                                   | 0.5 ± 0.1                                | 9.0                              | NC                                                  |
| 18:1(n-7)  | 0.6 ± 0.3                                   | 0.2 ± 0.04                               | 3.0                              | NC                                                  |
| 18:2(n-6)  | 1.4 ± 0.9                                   | 0.1 ± 0.04                               | 14                               | NC                                                  |
| 18:3(n-6)  | 0.4 ± 0.1                                   | 0.01 ± 0.01                              | 40                               | NC                                                  |
| 18:3(n-3)  | 0.05 ± 0.03                                 | ND                                       | NC                               | NC                                                  |
| 20:0       | 0.5 ± 0.2                                   | 0.02 ± 0.02                              | 25                               | NC                                                  |
| 20:1(n-9)  | 0.1 ± 0.04                                  | ND                                       | NC                               | NC                                                  |
| 20:3(n-6)  | ND                                          | 0.01 ± 0.002                             | NC                               | NC                                                  |
| 20:4(n-6)  | 0.1 ± 0.1                                   | 0.1 ± 0.1                                | 1.0                              | NC                                                  |
| 20:5(n-3)  | 0.3 ± 0.1                                   | ND                                       | NC                               | NC                                                  |
| 22:0       | 0.3 ± 0.1                                   | 0.1 ± 0.02                               | 3.0                              | NC                                                  |
| 24:0       | 0.4 ± 0.2                                   | 0.03 ± 0.01                              | 13                               | NC                                                  |
| Unsaturated| 8.0 ± 4.2                                   | 1.3 ± 0.3                                | 6.2                              | NC                                                  |
| Total      | 18 ± 7                                      | 5 ± 1                                    | 3.6                              | NC                                                  |

**FIGURE 4.** Higher concentration of total phosphatidylserine and phosphatidylserine containing unsaturated and short chain fatty acids in mitochondria relative to the whole cell in INS-1 832/13 cells and decreases in phosphatidylserines in mitochondria with glucose stimulation. Mitochondria were from the cells used in the experiments described in the legend to Fig. 3, as were whole cells. ND and NC, not detected and not calculated, respectively.

ules but higher than that in brain synaptic vesicles. The percentage of lysophosphatidylcholine (LPC) in ISG of 19% was about the same as that in chromaffin granules, much higher than that in brain synaptic vesicles (1%), and about twice as high as that in pancreatic granules (9%).

**Lipids, Percentage of Total**—Table 3 shows the percentages of individual fatty acids in the major PL classes in ISG, mitochondria, and whole INS-1 832/13 cells. The distribution of PL in ISG differed from that of the whole cell and mitochondrion. The percentage of short chain FA 12:0 and 14:0 in PS, PE, and PI in ISG and mitochondria were in general much higher than for the whole cell (Table 3). The percentage of unsaturated FA was about 25% higher in PS and PI compared with the whole cell. In contrast, the percentages in ISG and mitochondria of unsaturated FA in PE and PC were 15–30% lower than in the whole cell and equal to that in SM (Table 3).

The percentages of total FA 12:0 and 14:0 were much higher in PS, PE, PI, and SM compared with the whole cell. The percentage of FA 16:1 was lower in PS and PE in ISG compared with the whole cell. The percentage of FA 18:0 in PS, PE, and PI was much lower in ISG than in the whole cell. The percentage of FA 18:2(n-6) was higher in ISG PS and PI compared with the whole cell. The percentage of FA 20:4(n-6) in ISG was lower in PS, PE, and PI than in the whole cell, and the percentage in PC was higher. The percentage of FA 20:1(n-9) was higher in PS, PE, PI, PC, and SM in ISG than in the whole cell. The percentage of FA 22:1(n-9) in PS was much higher in ISG than in the mitochondrion and the whole cell. Overall, the percentage of total unsaturated PS, PE, and SM in ISG was similar to that of the whole cell. The percentage of unsaturated FA in PE and PC in ISG was less than half of the total percentage in the whole cell (Table 3). However, as shown in Figs. 3–14, the concentration of unsaturated FA in certain PL was much higher in ISG than in the whole cell.

**Granule PS**—The concentration of total PS in ISG, when expressed on the basis of the protein concentration, was 5-fold that of the whole cell. The concentration in ISG of PS containing unsaturated FAs was 5.8-fold that of the whole cell (Fig. 3). The levels of PS with the short chain FAs 12:0 and 14:0 were 8- and 23-fold higher than in whole cells. The PS with 16:0, 18:0, 18:1(n-7), and 18:1(n-9) FAs were present in levels 3–9-fold higher in ISG than in whole cells. All PS measured were more concentrated in the ISG fraction than in the whole cell fraction. The FAs present in the highest levels were 16:0, 18:0, and 18:1(n-9).

The levels of the total PS increased by 17% with glucose stimulation. PS containing unsaturated FAs increased 37%, and PS containing 12:0, 18:1(n-7)), 20:4(n-6), and 22:1(n-9) increased the most (Fig. 3). Interestingly, PS with 18:2(n-6) (linoleic acid), which is an essential FA that comes from the diet and is not produced in the body, was present at the low level of only 1.4 nmol/mg protein but concentrated 14-fold in ISG compared with the whole cell and doubled in ISG with glucose stimulation. The high content of short chain fatty acids and unsaturated fatty acids in ISG and their increase with glucose stimulation should make the ISG membranes more fluid.
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Mitochondrial PS—PS with various FA side chain lengths were concentrated more in mitochondria compared with the whole cell. However, the concentrations of the PS with shorter and medium chain FAs (12:0, 14:0, and 18:0) were one-third to one-half those in the ISG fractions (cf. Fig. 4 versus Fig. 3). The concentrations of the major FAs in PS 16:0, 18:0, 18:1(n-9), 18:1(n-7), and 18:2(n-6) were slightly lower or similar to those in the ISG fraction. The level of PS containing FAs of 20:0 length and longer were similar to those of the secretory granules, considering the probable lower accuracy of measurements of these lipids present at very low concentrations. The concentrations of almost all PS in the mitochondria (Fig. 4) decreased with glucose stimulation in contrast to the insulin granule fraction, where almost all PS increased (Fig. 3), possibly consistent with the conversion of PS to other PLs and their export out of mitochondria (55, 56) and a shift of PS out of mitochondria to the granules and elsewhere in the cell.

Granule PE—The concentration of total PE in ISG was essentially equal (1.2 fold) to its concentration in the whole cell (Fig. 5). The concentrations of PEs with 12:0 and 14:0 FAs were 50–50% lower in the ISG fraction than in the whole cell. The concentration of PEs with longer chain FAs in the granule were lower compared with those in the whole cells and in many cases undetectable. With glucose stimulation, the granule PEs with FA side chains 12:0, 14:0, and 16:0 decreased to 35–65% of the unstimulated granule PE levels. The concentrations of other granule PEs with longer FA chains were essentially unchanged with glucose stimulation, and the total decreased 35% (Fig. 5).

**TABLE 1.** The concentration of phosphatidylethanolamine in insulin secretory granules is similar to the whole cell in INS-1 832/13 cells, the concentration of phosphatidylethanolamine with short chain fatty acids is higher, and phosphatidylethanolamine in insulin secretory granules decreases with glucose stimulation. Measurements were made on the same insulin secretory granules and whole cells used in the experiments described in the legend to Fig. 3. ND and NC, not detected and not calculated, respectively.

| Fatty Acid | Unstimulated Granule (nmol/mg protein) | Unstimulated Whole Cell (nmol/mg protein) | Granule to Whole Cell Ratio | Percent Change vs Unstimulated Control Granule |
|------------|----------------------------------------|------------------------------------------|----------------------------|-----------------------------------------------|
| 12:0       | 1.0 ± 0.2                              | 0.04 ± 0.01                              | 25                         |                                               |
| 14:0       | 2.0 ± 0.5                              | 0.2 ± 0.02                               | 10                         |                                               |
| 16:0       | 26 ± 1.9                               | 7.1 ± 0.6                                | 3.7                        |                                               |
| 16:1(n-10) | 0.5 ± 0.2                              | 1.5 ± 0.2                                | 0.3                        |                                               |
| 18:0       | 27 ± 3.0                               | 26 ± 2.2                                 | 1.0                        |                                               |
| 18:1(n-9)  | 5.3 ± 1.2                              | 11 ± 1.1                                 | 0.5                        |                                               |
| 18:1(n-7)  | 2.0 ± 0.2                              | 2.8 ± 0.3                                | 0.7                        |                                               |
| 18:2(n-6)  | 0.04 ± 0.03                            | 1.3 ± 0.2                                | 0.03                       |                                               |
| 18:3(n-6)  | ND                                     | 0.1 ± 0.01                               | NC                         |                                               |
| 18:3(n-3)  | ND                                     | 0.03 ± 0.002                             | NC                         |                                               |
| 20:0       | 0.4 ± 0.1                              | 0.4 ± 0.03                               | 1.0                        |                                               |
| 20:1(n-7)  | 0.1 ± 0.1                              | ND                                       | NC                         |                                               |
| 20:1(n-9)  | 0.3 ± 0.03                             | 0.6 ± 0.2                               | 0.5                        |                                               |
| 20:3(n-6)  | 0.3 ± 0.1                              | 0.6 ± 0.1                                | 0.5                        |                                               |
| 20:3(n-9)  | 0.003 ± 0.003                          | ND                                       | NC                         |                                               |
| 20:4(n-6)  | 1.4 ± 0.6                              | 2.8 ± 0.3                                | 0.5                        |                                               |
| 20:5(n-3)  | ND                                     | 0.1 ± 0.005                              | NC                         |                                               |
| 22:0       | 0.02 ± 0.01                            | 0.2 ± 0.01                               | 0.1                        |                                               |
| 22:1(n-9)  | 0.1 ± 0.05                             | 0.2 ± 0.005                             | 0.5                        |                                               |
| 22:6(n-3)  | ND                                     | 0.4 ± 0.04                               | NC                         |                                               |
| 24:0       | 0.1 ± 0.04                             | 0.2 ± 0.004                             | 0.5                        |                                               |
| Unsaturated| 10 ± 2.5                               | 21 ± 2.5                                | 0.5                        |                                               |
| Total      | 67 ± 8                                 | 56 ± 5                                  | 1.2                        |                                               |

FIGURE 5. The concentration of phosphatidylethanolamine in insulin secretory granules is similar to the whole cell in INS-1 832/13 cells, the concentration of phosphatidylethanolamine with short chain fatty acids is higher, and phosphatidylethanolamine in insulin secretory granules decreases with glucose stimulation. Measurements were made on the same insulin secretory granules and whole cells used in the experiments described in the legend to Fig. 3. ND and NC, not detected and not calculated, respectively.

**TABLE 2.** The concentration of phosphatidylethanolamine with unsaturated fatty acids decreases 35% with glucose stimulation. Measurements were made on the same insulin secretory granules and whole cells used in the experiments described in the legend to Fig. 3. ND and NC, not detected and not calculated, respectively.
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Interestingly, with glucose stimulation the concentration of many of the PIs in the granule increased (Fig. 7), but the concentration of most PIs in the mitochondria decreased (Fig. 8). PIs in the whole INS-1 832/13 cells decrease with glucose stimulation (11). The decrease in PI levels in the whole cell is consistent with studies of pancreatic islets that show a breakdown of radiolabeled PIs with glucose stimulation performed years ago by our laboratory (38, 57, 58) and other laboratories (59–61). This might suggest a shift of phosphoinositides from other cellular compartments (such as the mitochondria, where the concentrations of most PIs decreased (Fig. 8)) into the insulin granule fraction with glucose stimulation.

Mitochondrial PI—The concentration of total PIs in mitochondria was about one-fifth as high as in the ISG and 68% that in the whole cell. PIs with FAs of 12:0, 14:0, and 16:1 were more concentrated in mitochondria compared with the whole cell, but about one-half or less as concentrated as in the ISG fraction. Other PIs in mitochondria except for those with FAs of 16:0, 18:0, 18:1(n-9), and 20:4(n-6) were slightly more concentrated in the mitochondria compared with the whole cell. As mentioned above, almost all mitochondrial PIs decreased with glucose stimulation (Fig. 8). The concentrations of PCs with various FAs were lower than in ISG or similar to the whole cell.

Granule PC—The concentrations of total PCs and PCs containing unsaturated FAs in ISG were 68 and 32%, respectively, that of whole cells (Fig. 9). The concentrations of PCs with 12:1, 20:4(n-6), and 20:5(n-3) were 26-, 3-, and 50-fold higher, respectively, in the ISG compared with the whole cells. PCs with 22:6(n-3) in ISG were present at a level of 0.21 nmol/mg of protein, which is 100-fold the level in the whole cell. The concentration of the total and unsaturated ISG PCs and most individual PCs did not increase significantly with glucose stimulation. Those showing a large percentage change were PCs present at extremely low levels, and thus the accuracy of their measured levels was low (Fig. 9).

Mitochondrial PC—The concentrations in mitochondria of total PCs and PCs with unsaturated FAs were 59 and 32%, respectively, that of the whole cell. The concentrations in mitochondria of the more abundant PCs with 14:0, 16:0, 18:0, and 18:1(n-9) FAs were 30–40% lower than in the ISG fraction, and the levels of the PCs with other FAs were very similar to those in the granule fraction. The concentration of PCs with FA chains 14:0, 16:0, and 18:0 were the same in mitochondria as in the whole cell. Except for 20:3(n-9), all 20 carbon FAs were higher in mitochondria than in the whole cell. PCs with 22:6(n-3) were present at a level of 0.4 nmol/mg of protein, which is a level 200-fold that in the whole cell. Except for PCs with 14:0 that were increased with glucose stimulation in mitochondria, similar to in insulin granule fractions, the changes in the PCs with other FAs were not significant. Any other large percentage changes were in PC containing a very low level of a FA, and thus the accuracy of their measured levels was low (Fig. 10).

Granule SMs—The concentrations of total SMs and SMs containing unsaturated FAs in ISG were 3.3- and 3.5-fold,
respectively, that in the whole cells. The concentration of SMs in ISG and whole cells was lower than that of the other PLs. The relative concentrations of SMs with individual FAs 12:0, 12:1, 14:0, 16:0, 16:1, and 18:0 were 3, 28, 12, 2.9, 7.7 and 2.5 in the ISG compared with those in the whole cells, respectively. The concentrations of SMs in the ISG were not increased with glu-

| Fatty Acid | Unstimulated Granule (nmol/mg protein) | Unstimulated Whole Cell (nmol/mg protein) | Granule to Whole Cell Ratio | Percent Change vs Unstimulated Control Granule |
|------------|----------------------------------------|-------------------------------------------|----------------------------|-----------------------------------------------|
| 12:0       | 2.2 ± 0.6                              | 0.1 ± 0.02                                | 22                         |                                               |
| 14:0       | 2.4 ± 0.6                              | 0.1 ± 0.03                                | 24                         |                                               |
| 16:0       | 14 ± 2.9                               | 2.2 ± 0.3                                 | 6.4                        |                                               |
| 16:1       | 1.2 ± 0.7                               | 0.4 ± 0.1                                 | 3.0                        |                                               |
| 18:0       | 9.9 ± 1.9                              | 4.5 ± 0.9                                 | 2.2                        |                                               |
| 18:1(n-9)  | 9.9 ± 3.7                              | 1.3 ± 0.3                                 | 7.6                        |                                               |
| 18:1(n-7)  | 1.2 ± 0.3                               | 0.5 ± 0.2                                 | 2.4                        |                                               |
| 18:2(n-6)  | 4.6 ± 1.1                               | 0.1 ± 0.02                                | 46                         |                                               |
| 18:3(n-6)  | 0.7 ± 0.6                               | 0.02 ± 0.01                               | 35                         |                                               |
| 18:3(n-3)  | 0.04 ± 0.04                             | ND                                        | NC                         |                                               |
| 20:0       | 0.5 ± 0.3                               | 0.03 ± 0.01                               | 17                         |                                               |
| 20:1(n-9)  | 0.1 ± 0.1                               | ND                                        | NC                         |                                               |
| 20:3(n-6)  | 0.1 ± 0.1                               | 0.03 ± 0.01                               | 3.3                        |                                               |
| 20:4(n-6)  | 0.1 ± 0.1                               | 0.5 ± 0.2                                 | 0.2                        |                                               |
| 20:5(n-3)  | 0.2 ± 0.1                               | ND                                        | NC                         |                                               |
| 22:0       | 0.3 ± 0.1                               | 0.1 ± 0.02                                | 3.0                        |                                               |
| 22:1(n-9)  | ND                                     | 0.1 ± 0.04                                | NC                         |                                               |
| 24:0       | 0.3 ± 0.2                               | 0.1 ± 0.02                                | 3.0                        |                                               |
| Unsaturated| 18 ± 6.8                                | 3.0 ± 0.9                                 | 6.0                        |                                               |
| Total      | 48 ± 13                                 | 10 ± 2                                    | 4.8                        |                                               |

**FIGURE 7.** Higher concentration of phosphatidylinositol and phosphatidylinositol containing unsaturated and short chain fatty acids in insulin secretory granules relative to the whole cell concentration in INS-1 832/13 cells and increases of phosphatidylinositols in insulin secretory granules with glucose stimulation. Measurements were made on the same insulin secretory granules and whole cells used in the experiments described in the legend to Fig. 3. ND and NC, not detected and not calculated, respectively.
Mitochondrial SM—The concentrations of total SMs and SMs containing unsaturated FAs in mitochondria were 1.7- and 1.6-fold, respectively, that in the whole cells and about half that of the ISG. Almost all SMs with individual FAs were concentrated in the mitochondria compared with the whole cell, but the concentrations were about half those of the ISG. Glucose stimulation did not change or lowered by 25% most of the SMs in mitochondria (Fig. 12).

Granule LPC—The concentrations in mitochondria of total LPCs and LPCs with unsaturated FAs were 2.8- and 4.3-fold, respectively, that of the whole cell. ISG LPCs with FAs 14:0, 16:0, 16:1, 18:0, and 18:1(n-9) ranged from 1.7- to 4.7-fold that of the whole cell. The levels of total, unsaturated, and many individual LPCs decreased 40–60% with glucose stimulation (Fig. 13).

Mitochondria LPC—The concentrations of total and unsaturated LPCs in mitochondria were 30 and 40%, respectively, those in the whole cell. These, as well as individual LPCs, decreased 20% to almost 50% with glucose stimulation (Fig. 14).

DISCUSSION

The profile in the beta cell of the enzymes necessary for lipid synthesis strongly suggests that the beta cell is a lipogenic tissue (11). Lipids increase in the beta cell with glucose stimulation during a time course that coincides with insulin secretion (11). Also, knockdown of FAS inhibits the glucose-stimulated increase of lipids and inhibits insulin release (12). It would seem, based on a priori reasoning, that rapid lipid remodeling of intracellular membranes is especially necessary during insulin synthesis and exocytosis, when proinsulin is synthesized in the endoplasmic reticulum and converted to insulin as it traverses the cis- and trans-Golgi networks and is packaged into secretory granules, and the granule membranes are modified as granules mature until eventually the insulin granule membranes fuse with the plasma membrane, where insulin is finally extruded into the circulation. Synthesis of precursors of lipids (i.e. short chain acyl-CoAs) by mitochondria may play a role in the lipid restructuring of cellular membranes because knockdown of the mitochondrial enzymes pyruvate carboxylase (23, 24), needed for anaplerosis/cataplerosis, and succinyl-CoA:3-ketoacid-CoA transferase (28), needed for the acetoacetate pathway (Fig. 1), inhibits glucose-stimulated insulin release...
and prevents the increase in PLs in the beta cells (12). The current work shows that ISG are a major site of phospholipid remodeling in glucose-stimulated beta cells. PLs, the major lipids in cellular membranes, especially the negatively charged ones and those with unsaturated FAs, are concentrated in ISG and increase with glucose stimulation (Figs. 2, 3, 5, 7, 9, 11, and 13).

**Lipid Composition of ISG**—A high lipid/protein ratio is a general characteristic of secretory granule and vesicle membranes (52–54), such as chromaffin granules, brain synaptic vesicles, and pancreas exocrine granule membranes (54), and this is what we observed with ISG. The concentration of many PLs in ISG from INS-1 832/13 cells is much higher than their concentrations in the whole cell or mitochondria (Figs. 3, 7, 11, and 13). As discussed below, the negatively charged PLs PS and PI in ISG are most likely the PLs most relevant to docking of ISG with the plasma membrane during insulin exocytosis. The concentrations of PS (Fig. 3), PI (Fig. 7), and SM (Fig. 11) in ISG were 5-, 5-, and 3-fold, respectively, that of the whole cell. In most tissues, PS is abundant in the plasma membrane and certain intracellular organelles. Table 2 shows the percentages of various PLs in ISG compared with values published for other types of neurosecretory granules. The percentage of PS (10%) in ISG is very similar to the percentage of PS in bovine, rat, and human chromaffin granules (9–11%) and brain synaptic vesicles (12%) and much higher than in pancreatic granules (2%), whereas the percentages of PE and PC in ISG are about 30% lower than in chromaffin granules, pancreatic granules, and brain synaptic vesicles. The percentage of PI in ISG (21%) is much higher than in chromaffin granules and brain synaptic vesicles (1–3%) but is about the same as in pancreatic granules (15%) (53, 54). The percentage of LPC in ISG (19%) is very similar to the percentage in chromaffin granules but much higher than the percentage in brain synaptic vesicles (1%). The percentage of SM among lipid classes in ISG (10%) is within the range in chromaffin granules and pancreatic granules (9–16%) but higher than in brain synaptic vesicles (5%) (48–54).

**Phospholipids in Vesicle Membranes Relative to Other Cellular Membranes**—Substantial information is known about PLs in whole cells and the plasma membrane as well as in vesicles in many cell types but not in ISG. Knowledge of the lipid composition and function of vesicles has lagged behind the knowledge of proteins in intracellular organelles, especially in ISG that have not previously been systematically studied. The FA composition of PL in the ISG relates to the idea of asymmetric distribution of lipid classes across a bilayer. Although there are conflicting data, according to the universal model membrane based on the plasma membrane of human erythrocytes and

![Table showing phosphatidylcholine concentrations in ISG and whole cells](image-url)
other tissues, PS, PE, and PI are located on the cytoplasmic side of the plasma membrane, and most PC and SM are on the outer surface (55). In almost all mammalian cells, PC accounts for 50% of total cellular phospholipids. PE is the second most abundant phospholipid in mammalian membranes, contributing 20–30% of total phospholipid content. PE is found in greater abundance in the inner leaflet of the plasma membrane, with ~20% of plasma membrane PE found on the outer leaflet. Due to the small headgroup of PE, it has a propensity to form non-bilayer structures. This is important in the formation of new membranes and vesicles as well as for membrane fusion and budding processes. According to the universal membrane model, this would place PC and SM on the luminal side of the ISG membrane and PS, PE, and PI on the cytoplasmic side of the ISG membrane. This puts stable bilayer formers on the luminal surface and the lipids most favoring membrane fluidity on the cytoplasmic surface (52).

PS is the most abundant anionic PL, and it accounts for 5–10% of cellular PL in most cells. The predominance of PS in the cytosolic (inner) monolayer surface of the plasma membrane and its tendency to be located on the cytosolic (outer) leaflet of intracellular vesicles (55) have implications for protein secretion. The anionic character of the serine headgroup of PS promotes interactions between PS and the positively charged SNARE proteins in the inner leaflet of the plasma membrane and the positively charged SNARE proteins in the outer leaflet of vesicles to facilitate contact between these proteins during secretion of the contents of the vesicles. In addition, PS is an essential cofactor that binds to and activates a large number of proteins especially those with signaling activities (55), including some involved with protein secretion (62–68).

**PL Composition in ISG Relevant to Membrane Fluidity, Curvature, and Fusion**—The properties of PL in ISG suggest that they were designed for fusion of the ISG bilayer with the plasma membrane bilayer. Conditions that favor membrane fusion are increased membrane fluidity and curvature. Membrane fluidity is favored by short chain and unsaturated FA in PL. Unsaturated FA can also increase the curvature of membranes. ISG are particularly rich in PS, PI, and SM compared with these PL classes in the whole cell and mitochondria (Figs. 3, 7, and 11 and Tables 2 and 3). The ISG content of the shorter chain fatty acids 12:0 and/or 14:0 are quite high in PS, PE, PI, and SM compared with these PL classes in the whole cell and also compared with the mitochondria (Figs. 3, 5, 7, and 11).

The concentration of unsaturated fatty acids is quite high in ISG compared with the whole cell and/or mitochondria (Figs. 3, 7, 11, and 13). The concentration of unsaturated FA in PS and PI (ISG PS and PI compared with whole cell unsaturated FA ratios of 5.8 and 6 in PS and PI) is favored by short chain and unsaturated FA in PL. Unsaturated FA can also increase the curvature of membranes. ISG are particularly rich in PS, PI, and SM compared with these PL classes in the whole cell and also compared with the mitochondria (Figs. 3, 5, 7, and 11).

**FIGURE 11.** Higher concentration of total sphingomyelin and sphingomyelin containing unsaturated and short chain fatty acids in insulin secretory granules relative to the whole cell in INS-1 832/13 cells and no changes in sphingomyelin in insulin secretory granules with glucose stimulation. Measurements were made on the same insulin secretory granules and whole cells used in the experiments described in the legend to Fig. 3. ND and NC, not detected and not calculated, respectively.
containing unsaturated fatty acids increased by 28 and 37%, respectively, with glucose stimulation (Figs. 3 and 7). With glucose stimulation, the concentration of ISG PS with fatty acid chains 12:0, 18:1(n-7), 18:2(n-6), 18:3(n-6), 20:4(n-6), and 22:1(n-9) increased. Among the PI in the ISG, almost all of them increased with glucose stimulation (Fig. 7), whereas the phosphoinositides in mitochondria (Fig. 8) and the in the whole cell decreased (11). This may indicate a breakdown of PI in the whole cell, as is known to occur with glucose stimulation in pancreatic islets.
(38, 57–61) and in addition an intracellular shift of PI into the ISG with glucose stimulation. The concentrations of PC and SM in the ISG where not changed significantly with glucose stimulation except for an increase in 14:0 in the PC (Fig. 9).

Glutamine is a metabolic fuel that can supply energy for cells in tissue culture, including in beta cells. Because the cells were maintained in complete tissue culture medium that contains glutamine during the entire duration of the experiments in the presence and absence of glucose, any changes with glucose compared with the absence of glucose were a result of stimulation by glucose rather than glucose acting only as a fuel.

We are aware of only one other study of PL in insulin granules. This study was focused on arachidonic acid in PLs and plasmalogens. Plasmalogens are a type of PL with an ether linkage at the sn-2 position and fatty alcohol palmitic, oleic, or stearic residues at the sn-1 position as their main that reduce the VAMP2 net positive charge and thus its interaction with negatively charged PS to act as a coupling factor, facilitating interaction between the polycatonic positive charged binding domains of Vamp2 in the vesicles and Syx1a and SNAP25 located in the plasma membrane (67). This facilitates the “zippering” interaction between the two membranes and thus the docking of the vesicle and fusion with the plasma membrane. In support of this concept, mutations in the lipid-binding juxtamembrane domain that reduce the VAMP2 net positive charge and thus its interaction with negatively charged lipids interfere with normal catecholamine release from chromaffin cells (72, 75) and inhibit secretion of insulin granules from beta cells (68). In line with this idea that PS and PI enhance the fusion of the vesicle membrane with the target plasma membrane, we observed that the concentrations of negatively charged PS and PI are 5-fold higher in ISG compared with the whole cell and that upon glucose stimulation, the concentrations of PS and PI increased in the ISG (Figs. 3 and 7).

PS could play additional roles that facilitate the docking of ISG with the plasma membrane. The synaptic vesicle protein synaptotagmin 1 (Syt1) binds PS in a calcium-dependent manner (64, 65). Increased PS levels in PC12 cells produce an increase in calcium-triggered membrane fusion and catecholamine release (62, 63). PS may enhance membrane fusion.

**Possible Interactions of ISG PL with SNARE Proteins**—Evidence from many studies of models of membrane-membrane fusion suggests that vesicle PS plays a specific role in exocytosis via its interaction with vesicle and plasmalemmal SNARE proteins that carry out the docking and fusion of the vesicle membrane with the target plasma membrane. In neurons and neurosecretory cells, regulated exocytosis requires calcium-dependent fusion of the membrane of a docked vesicle or secretary granule aided by SNARE proteins. In synaptic vesicles, a much studied model, this fusion is mediated by the SNARE protein VAMP2 (also called synaptobrevin2 (Syb2)) anchored in the outer leaf of the vesicle membrane interacting in a zipper-like fashion with syntaxin1a (Syx1a) and SNAP25 anchored in the target plasma membrane (68, 71–75). These proteins are also found in the beta cell (68, 77–83).

SNARE proteins Syx1a, VAMP2, and SNAP25 share 60–70-amino acid-long highly conserved juxtamembrane polybasic amino acid sequences called SNARE motifs that bind negatively charged PL, such as PS. Syx1a and VAMP2 possess a single SNARE motif, and SNAP25 possesses two such domains (74). The enrichment of acidic PL, such as PS and PI (72, 76), in the inner leaflet of the plasma membrane and the presence of PS on the outer leaflet of vesicle membranes allows the negatively charged PS to act as a coupling factor, facilitating interaction between the polycatonic positive charged binding domains of Vamp2 in the vesicles and Syx1a and SNAP25 located in the plasma membrane (67). This facilitates the “zippering” interaction between the two membranes and thus the docking of the vesicle and fusion with the plasma membrane. In support of this concept, mutations in the lipid-binding juxtamembrane domain that reduce the VAMP2 net positive charge and thus its interaction with negatively charged lipids interfere with normal catecholamine release from chromaffin cells (72, 75) and inhibit secretion of insulin granules from beta cells (68). In line with this idea that PS and PI enhance the fusion of the vesicle membrane with the target plasma membrane, we observed that the concentrations of the negatively charged PS and PI are 5-fold higher in ISG compared with the whole cell and that upon glucose stimulation, the concentrations of PS and PI increased in the ISG (Figs. 3 and 7).
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through an affinity for curved areas of membrane vesicles. A 25-mer peptide, MARCKS-ED, based on the effector domain sequence of the intracellular membrane protein myristoylated alanine-rich protein kinase C substrate, can recognize PS with preferences for highly curved vesicles (66).

Active Role of Flippase Enzymes in Insulin Secretion—Flip- pases are P4 ATPases that rapidly move the aminophospholipids PS and PE from the outer leaflet to the cytosolic leaflet of the plasma membrane and, in the case of intracellular vesicles, from the inner leaflet to the cytosolic leaflet (84 – 87). These integral membrane proteins transfer the polar head of the aminophospholipid through the hydrophobic center of the lipid bilayer against a concentration gradient. From the findings discussed above, one can see that the movement of PS from the inner to the outer leaflet of the ISG could facilitate docking of the ISG with the plasma membrane. We have identified the flippases present in human and rat in pancreatic islets and INS-1 832/13 cells, including in ISG. Knockdown of these flippases in INS-1 832/13 cells and human pancreatic islets inhibits glucose-stimulated insulin release.3

Conclusion—The current data show that ISG PLs, especially negatively charged PLs PS and PI, as well as PLs containing short chain and unsaturated fatty acids are in a dynamic state during insulin exocytosis. Taken together with known actions of PS and phosphoinositides as well as properties of PLs containing short chain and unsaturated fatty acids in enhancing fusion of lipid bilayers, the results indicate that ISG PL modifications play key roles in the insulin exocytosis process. Flippase data further suggest that the PLs of the ISG are rapidly modified and are in a dynamic state during insulin secretion.

Note Added in Proof—Israr H. Ansari’s contributions to this article fulfill the JBC authorship criteria, but his authorship was inadvertently omitted from the version of the article that was published on March 11, 2015 as a Paper in Press.

REFERENCES

1. Corkey, B. E., Deeney, J. T., Yaney, G. C., Tornheim, K., and Prentki, M. (2000) The role of long-chain fatty acyl-CoA esters in beta cell signal transduction. *J. Nutr.* 130, 2995–3045

2. Farfari, S., Schulz, V., Corkey, B., and Prentki, M. (2000) Glucose-regulated anaplerosis and cataplerosis in pancreatic beta-cells: possible implication of a pyruvate/citrate shuttle in insulin secretion. *Diabetes* 49, 718–726

3. Flamez, D., Berger, V., Kruhoffer, M., Orntoft, T., Pipeleers, D., and Schuit, F. C. (2002) Critical role for cataplerosis via citrate in glucose-regulated insulin release. *Diabetes* 51, 2018–2024

4. MacDonald, M. J., Fahien, L. A., Brown, L. J., Hasan, N. M., Buss, J. D., and Kendrick, M. A. (2005) Perspective: emerging evidence for signaling roles of mitochondrial anaplerotic products in insulin secretion. *Am. J. Physiol. Endocrinol. Metab.* 288, E1–E15

5. MacDonald, M. J., Smith, A. D., 3rd, Hasan, N. M., Sabat, G., and Fahien, L. A. (2007) Feasibility of pathways for transfer of acyl groups from mitochondria to the cytosol to form short chain acyl CoAs in the pancreatic beta cell. *J. Biol. Chem.* 282, 30596–30606

6. MacDonald, M. J. (2007) Synergistic potent insulin release by combinations of weak secretagogues in pancreatic islets and INS-1 cells. *J. Biol. Chem.* 282, 6043–6052

7. Muoio, D. M., and Newgard, C. B. (2008) Mechanisms of disease: molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. *Nat. Rev. Mol. Cell Biol.* 9, 193–205

8. Jitrapakdee, S., Wuthisathapornchai, A., Wallace, J. C., and MacDonald, M. J. (2010) Regulation of insulin secretion: role of mitochondrial signaling. *Diabetologia* 53, 1019–1032

9. Prentki, M., Matschinsky, F. M., and Madiraju, S. R. (2013) Metabolic signaling in fuel-induced insulin secretion. *Cell Metab.* 18, 162–185

10. Lorenz, M. A., El Azzouny, M. A., Kennedy, R. T., and Burant, C. F. (2013) Metabolome response to glucose in the β-cell line INS-1 832/13. *J. Biol. Chem.* 288, 10923–10935

11. MacDonald, M. J., Hasan, N. M., Dobrzyn, A., Stoker, S. W. (2008) The role of rapid lipogenesis in insulin secretion: insulin secretagogues acutely alter lipid composition of INS-1 832/13 cells. *Arch. Biochem. Biophys.* 470, 153–162

12. MacDonald, M. J., Hasan, N. M., Dobrzyn, A., Stoker, S. W., Ptito, V., and Poukitus, P. E., (2011) Knockdown of mitochondrial anaplerotic products in insulin secretion. *Diabetes* 60, 315–321

13. Prentki, M., and Madiraju, S. R. (2012) Glycerolipid/free fatty acid cycle and islet β-cell function in health, obesity and diabetes. *Mol. Cell Endocrinol.* 353, 88–100

14. Prentki, M. (2011) Fuel-stimulated insulin secretion depends upon mitochondrial signal and future. *J. Inherit. Metab. Dis.* 29, 327–331

15. Cline, G. W. (2011) Fuel-stimulated insulin secretion depends upon mitochondrial activation and the integration of mitochondrial and cytosolic substrate cycles. *Diabetes Metab. J.* 35, 458–465

16. Cline, G. W., Pongratz, R. L., Zhao, X., and Papas, K. K. (2011) Rates of insulin secretion in INS-1 cells are enhanced by coupling to anaplerosis and Kreb’s cycle flux independent of ATP synthesis. *Biochem. Biophys. Res. Commun.* 415, 30–35

17. Pante, Y., Willenborg, M., Schumacher, K., Hamada, A., Ghaly, H., and Rustenbeck, I. (2013) Acute metabolic amplification of insulin secretion in mouse islets is mediated by mitochondrial export of metabolites, but not by mitochondrial energy generation. *Metabolism* 62, 1375–1386

18. MacDonald, M. J., Longacre, M. J., Warner, T. F., and Thonpho, A. (2013) Glucose-regulated cataplerosis and insulin secretion in insulinoma cells caused by siRNA-mediated suppression of pyruvate carboxylase. *Diabetes* 62, 294, E1287–E1297

19. Cline, G. W. (2011) Fuel-stimulated insulin secretion depends upon mitochondrial activation and the integration of mitochondrial and cytosolic substrate cycles. *Diabetes Metab. J.* 35, 458–465

20. Cline, G. W., Pongratz, R. L., Zhao, X., and Papas, K. K. (2011) Rates of insulin secretion in INS-1 cells are enhanced by coupling to anaplerosis and Kreb’s cycle flux independent of ATP synthesis. *Biochem. Biophys. Res. Commun.* 415, 30–35

21. Pante, Y., Willenborg, M., Schumacher, K., Hamada, A., Ghaly, H., and Rustenbeck, I. (2013) Acute metabolic amplification of insulin secretion in mouse islets is mediated by mitochondrial export of metabolites, but not by mitochondrial energy generation. *Metabolism* 62, 1375–1386

22. MacDonald, M. J., Longacre, M. J., Warner, T. F., and Thonpho, A. (2013) High level of ATP citrate lyase expression in human and rat pancreatic islets. *Horm. Metab. Res.* 45, 391–393

23. Hasan, N. M., Longacre, M. J., Stoker, S. W., Boonsaen, T., Jitrapakdee, S., Kendrick, M. A., Wallace, J. C., and MacDonald, M. J. (2008) Impaired anaplerosis and insulin secretion in insulinaoma cells caused by siRNA-mediated suppression of pyruvate carboxylase. *J. Biol. Chem.* 283, 28048–28059

24. Xu, J., Han, J., Long, Y. S., Epstein, P. N., and Liu, Y. Q. (2008) The role of pyruvate carboxylase in insulin secretion and proliferation in rat pancreatic beta cells. *Diabetesologia* 51, 2022–2030

25. MacDonald, M. J., Longacre, M. J., Stoker, S. W., Brown, L. J., Hasan, N. M., and Kendrick, M. A. (2008) Acetoacetate and β-hydroxybutyrate in combination with other metabolites release insulin from INS-1 cells and provide clues about pathways in insulin secretion. *Am. J. Physiol. Cell Physiol.* 294, C442–C450

26. MacDonald, M. J., Hasan, N. M., and Longacre, M. J. (2008) Studies with leucine, β-hydroxybutyrate and ATP citrate lyase-deficient cells support the acetoacetate pathway of insulin secretion. *Biochim. Biophys. Acta* 1780, 966–972

27. MacDonald, M. J., Stoker, S. W., and Hasan, N. M. (2008) Anaplerosis from glucose, α-ketoisocaproate, and pyruvate in pancreatic islets, INS-1
Insulin Granule Phospholipids in Pancreatic Beta Cells

and purification. *Can. J. Biol. Chem.* 37, 911–917

46. Dobrzyn, A., Dobrzyn, P., Miyazaki, M., Sampath, H., Chu, K., and Ntambi, J. M. (2005) Stearoyl-CoA desaturase 1 deficiency increases CTP:choline cytidylyltransferase translocation into the membrane and enhances phosphatidylcholine synthesis in liver. *J. Biol. Chem.* 280, 23356–23362

47. MacDonald, M. J. (1981) High content of mitochondrial glycerol-3-phosphate dehydrogenase in pancreatic islets and its inhibition by diazoxide. *J. Biol. Chem.* 256, 8287–8290

48. Breckenridge, W. C., Gombos, G., and Morgan, I. G. (1972) The lipid composition of adult rat brain synaptosomal plasma membranes. *Biochim. Biophys. Acta* 266, 695–707

49. Breckenridge, W. C., Morgan, I. G., Zanetta, J. P., and Vincendon, G. (1973) Adult rat brain synaptic vesicles. II. Lipid composition. *Biochim. Biophys. Acta* 320, 681–686

50. Winkler, H. (1976) The composition of adrenal chromaffin granules: an assessment of controversial results. *Neuroscience* 1, 65–80

51. Grewe, B. K. (1998) Gangliosides and phospholipids of the membranes from bovine adrenal medullary chromaffin granules. *Biochim. Biophys. Acta* 489, 89–97

52. Westhead, E. W. (1987) Lipid composition and orientation in secretary vesicles. *Annu. N.Y. Acad. Sci.* 493, 92–100

53. Castle, J. D., Cameron, R. S., Arvan, P., von Zastrow, M., and Rudnick, G. (1987) Similarities and differences among neuroendocrine, exocrine, and endocytic vesicles. *Annu. N.Y. Acad. Sci.* 493, 448–460

54. Thiele, C., and Huttner, W. B. (1998) Protein and lipid sorting from the trans-Golgi network to secretory granules: recent developments. *Semin. Cell Dev. Biol.* 9, 511–516

55. Vance, D. E., and Vance, J. E. (2008) In *Biochemistry of Lipids, Lipoproteins and Membranes*, 5th Ed. (D. E. Vance, J. E., eds) pp. 213–244, Elsevier, Amsterdam

56. Vance, J. E., and Tasseva, G. (2013) Formation and function of phosphatidylinerse and phosphatidylethanolamine in mammalian cells. *Biochim. Biophys. Acta* 1831, 543–554

57. MacDonald, M. J. (1985) Evidence for glucose-responsive and -unresponsive pools of phospholipid in pancreatic islets. *J. Biol. Chem.* 260, 7861–7867

58. Zhang, Z., Hui, E., Chapman, E. R., and Jackson, M. B. (2009) Phosphatidylethanolamine phospholipase C is involved in calcium signaling of inositol phosphates in pancreatic islets. *J. Biol. Chem.* 284, 31592–31600

59. Turk, J., Wolf, B. A., Lefkowith, J. B., Stump, W. T., and McDaniel, M. L. (1987) Potentiation by glucose metabolites of inositol trisphosphate-inhibited calcium mobilization in isolated pancreatic islets. Predominance of inositol phosphates in permeabilized rat pancreatic islets. *J. Biol. Chem.* 262, 13567–13570
tein localization. Science 319, 210–213
68. Williams, D., Vicogne, J., Zaitseva, I., McLaughlin, S., and Pessin, J. E. (2009) Evidence that electrostatic interactions between vesicle-associated membrane protein 2 and acidic phospholipids may modulate the fusion of transport vesicles with the plasma membrane. Mol. Biol. Cell 20, 4910–4919
69. Ramanadham, S., Bohrer, A., Gross, R. W., and Turk, J. (1993) Mass spectrometric characterization of arachidonate-containing plasmalogens in human pancreatic islets and in rat islet beta-cells and subcellular membranes. Biochemistry 32, 13499–13509
70. Ramanadham, S., Hsu, F. F., Bohrer, A., Nowatzke, W., Ma, Z., and Turk, J. (1998) Electrospray ionization mass spectrometric analyses of phospholipids from rat and human pancreatic islets and subcellular membranes: comparison to other tissues and implications for membrane fusion in insulin exocytosis. Biochemistry 37, 4553–4567
71. Wang, T., Smith, E. A., Chapman, E. R., and Weisshaar, J. C. (2009) Lipid mixing and content release in single-vesicle, SNARE-driven fusion assay with 1–5 ms resolution. Biophys. J. 96, 4122–4131
72. de Haro, L., Ferracci, G., Opi, S., Iborra, C., Quetglas, S., Miquelis, R., Lévèque, and Seagar, M. (2004) Ca\textsuperscript{2+}/calmodulin transfers the membrane-proximal lipid-binding domain of the v-SNARE synaptobrevin from cis to trans bilayers. Proc. Natl. Acad. Sci. U.S.A. 101, 1578–1583
73. Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature 362, 318–324
74. Domanska, M. K., Kiessling, V., and Tammi, L. K. (2010) Docking and fast fusion of synaptobrevin vesicles depends on the lipid compositions of the vesicle and the acceptor SNARE complex-containing target membrane. Biophys. J. 99, 2936–2946
75. Quetglas, S., Iborra, C., Sasakawa, N., De Haro, L., Kumakura, K., Sato, K., Lévèque, C., and Seagar, M. (2002) Calmodulin and lipid binding to synaptobrevin regulates calcium-dependent exocytosis. EMBO J. 21, 3970–3979
76. Kooijman, E. E., King, K. E., Gangoda, M., and Gericie, A. (2009) Ionization properties of phosphatidylinositol polyphosphates in mixed model membranes. Biochemistry 48, 9360–9371
77. Wheeler, M. B., Sheu, L., Ghai, M., Bouquillon, A., Grondin, G., Weller, U., Beaudoin, A. R., Bennett, M. K., Trimble, W. S., and Gaisano, H. Y. (1996) Characterization of SNARE protein expression in beta cell lines and pancreatic islets. Endocrinology 137, 1340–1348
78. Thurmond, D. C. (2000) Regulation of insulin action and insulin secretion by SNARE-mediated vesicle exocytosis. In Madame Curie Bioscience Database, Landes Bioscience, Austin, TX
79. Thurmond, D. C., Gonelle-Gispert, C., Furukawa, M., Halban, P. A., and Pessin, J. E. (2003) Glucose-stimulated insulin secretion is coupled to the interaction of actin with the t-SNARE (target membrane soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein) complex. Mol. Endocrinol. 17, 732–742
80. Oh, E., Kalwat, M. A., Kim, M. J., Verhage, M., and Thurmond, D. C. (2012) Munc18–1 regulates first-phase insulin release by promoting granule docking to multiple syntaxin isoforms. J. Biol. Chem. 287, 25821–25833
81. Wang, Z., and Thurmond, D. C. (2009) Mechanisms of biphasic insulin-granule exocytosis: roles of the cytoskeleton, small GTPases and SNARE proteins. J. Cell Sci. 122, 893–903
82. Daniel, S., Noda, M., Straub, S. G., and Sharp, G. W. (1999) Identification of the docked granule pool responsible for the first phase of glucose-stimulated insulin secretion. Diabetes 48, 1686–1690
83. Jacobsson, G., Bean, A. J., Scheller, R. H., Jungti-Berggren, L., Deeney, J. T., Berggren, P. O., and Meister, B. (1994) Identification of synaptic proteins and their isoform mRNAs in compartments of pancreatic endocrine cells. Proc. Natl. Acad. Sci. U.S.A. 91, 12487–12491
84. Daleke, D. L. (2007) Phospholipid flippases. J. Biol. Chem. 282, 821–825
85. Clark, M. R. (2011) Flippin’ lipids. Nat. Immunol. 12, 373–375
86. Devaux, P. F., Herrmann, A., Ohlwein, N., and Kozlov, M. M. (2008) How lipid flippases can modulate membrane structure. Biochim. Biophys. Acta 1778, 1591–1600
87. van der Mark, V. A., Elferink, R. P., and Paulusma, C. C. (2013) P4 ATPases: flippases in health and disease. Int. J. Mol. Sci. 14, 7897–7922