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Secretagogue-Induced Diacylglycerol Accumulation in Isolated Pancreatic Islets. Mass Spectrometric Characterization of the Fatty Acyl Content Indicates Multiple Mechanisms of Generation†

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ABSTRACT: Diacylglycerol accumulation has been examined in secretagogue-stimulated pancreatic islets with a newly developed negative ion chemical ionization mass spectrometric method. The muscarinic agonist carbachol induces islet accumulation of diacylglycerol rich in arachidonate and stearate, and a parallel accumulation of ^{3}H-labeled diacylglycerol occurs in carbachol-stimulated islets that had been prelabeled with [^{3}H]glycerol. Islets so labeled do not accumulate ^{3}H-labeled diacylglycerol in response to D-glucose, but D-glucose does induce islet accumulation of diacylglycerol by mass. This material is rich in palmitate and oleate and contains much smaller amounts of arachidonate. Neither secretagogue influences triacylglycerol labeling, and neither induces release of [^{3}H]choline or [^{3}H]phosphocholine from islets prelabeled with [^{3}H]choline. These observations indicate that the diacylglycerol that accumulates in islets in response to carbachol arises from hydrolysis of glycerolipids, probably including phosphoinositides. The bulk of the diacylglycerol which accumulates in response to glucose does not arise from glycerolipid hydrolysis and must therefore reflect de novo synthesis. The endogenous diacylglycerol which accumulates in secretagogue-stimulated islets may participate in insulin secretion because exogenous diacylglycerol induces insulin secretion from islets, and an inhibitor of diacylglycerol metabolism to phosphatidic acid augments glucose-induced insulin secretion.

The biochemical events which regulate insulin secretion from the β cells of pancreatic islets are at present incompletely understood. Two general classes of insulin secretagogues are recognized (Wollheim & Sharp, 1981; Prentki & Matschinsky, 1987). One class induces secretion by interaction with β-cell plasma membrane receptors. Muscarinic agonists including acetylcholine and carbachol are examples of insulin secretagogues from this class. A second class of insulin secretagogues is metabolized by glycolytic and/or tricarboxylic acid pathways in islets and may induce secretion by virtue of their utilization as fuels (Malaisse et al., 1979; Hedeskov, 1980; Ashcroft, 1980; Meglasson & Matschinsky, 1986). D-Glucose is an example of such a fuel secretagogue and is the predominant physiologic regulator of insulin secretion. Elucidation of the biochemical mechanisms underlying glucose-induced insulin secretion remains an important objective because there is a relatively selective impairment in glucose-induced insulin secretion in the disease type II diabetes mellitus despite nearly normal insulin secretory responses to some other secretagogues (Pfeiffer et al., 1981).

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Phospholipid-derived mediator substances are known to accumulate in glucose-stimulated islets and may serve as messengers in stimulus–response coupling in the β cell (Prentki & Matschinsky, 1987; Turk et al., 1987). Such compounds include arachidonic acid (Wolf et al., 1986) and its metabolites (Metz et al., 1983; Turk et al., 1984a,b; Metz, 1985) and inositol trisphosphate (Morgan et al., 1985; Turk et al., 1986; Biden et al., 1987). Inositol trisphosphate accumulation is also induced by muscarinic agonists both in islet cells and in clonal insulin-secreting cells (Wollheim & Biden, 1986; Biden et al., 1987; Wolf et al., 1988). Production of inositol phosphates involves hydrolysis of phosphoinositides by phospholipase(s) of the C type (Berridge, 1987). The other product of this reaction is diacylglycerol. Diacylglycerol is thought to serve, among other functions, as an endogenous activator of the regulatory enzyme protein kinase C (Kikkawa & Nishizuka, 1986). Diacylglycerols also directly influence the physical properties of phospholipid bilayers (Epand, 1985) and the activity of other enzymes by processes independent of protein kinase C (Goppelt-Strube et al., 1987; Burch, 1988; Kolesnick & Clegg, 1988). Diacylglycerol accumulation has been proposed, on the basis of indirect evidence, to govern the second phase of glucose-induced insulin secretion (Zawalich et al., 1983) and to participate in the induction of memory in β cells (Zawalich, 1988; Zawalich et al., 1988; Niki et al., 1988).

There is relatively little direct evidence on the influence of insulin secretagogues on islet diacylglycerol content or on the contribution of several potential sources to islet diacylglycerol production. Such sources could include hydrolysis of either inositol or choline phospholipids. The former would yield a diacylglycerol rich in arachidonate and stearate (Turk et al., 1986b; Holub & Kuskis, 1978). Diacylglycerol from the latter source would contain substantial amounts of palmitate and oleate and would be produced simultaneously with phosphocholine (Boccking et al., 1985; Irving & Exton, 1987; Exton, 1988). Other sources of islet diacylglycerol could include triacylglycerol hydrolysis (Banschbach et al., 1981; Soling et al., 1987) and de novo synthesis (Dunlop & Larkins, 1985a,b; Farese et al., 1986; Vara & Tamarit-Rodriguez, 1986). Glucose has been reported to induce the accumulation of ^{3}H-labeled diacylglycerol in islets prelabeled with [^{3}H]glycerol in some studies (Montague & Parkin, 1980) but not in others (Peter-Riesch et al., 1988). Glucose has also been reported to induce accumulation of arachidonoyl–diacylglycerol species in islets in some studies (Dunlop & Larkins, 1984), but not in others (Peter-Riesch et al., 1988). To further examine those issues, we have developed a negative ion chemical ionization mass spectrometric method for determination of the fatty acid composition of trace quantities of glycerolipids and have used this method to quantitate diacylglycerol accumulation in secretagogue-stimulated pancreatic islets.

Experimental Procedures

Materials. Male Sprague-Dawley rats (180–200-g body weight) were purchased from Sasco (O'Fallon, MO). Collagenase (CLS IV) was obtained from Cooper Biochemical (Freehold, NJ) or Boehringer Mannheim (Indianapolis, IN). Tissue culture medium (CMRL-1066), penicillin, streptomycin, Hanks' buffer, heat-inactivated fetal bovine serum, and L-glutamine were from Gibco (Grand Island, NY). Pentex bovine serum albumin (fatty acid free, fraction V) was obtained from Miles Laboratories (Elkhart, IN). Rodent chow 5001 was purchased from Ralston Purina (St. Louis, MO). Standard fatty acids were obtained from NuChek Prep (Elysian, MN). Standard phospholipids were from Sigma Chemical (St. Louis, MO). D-Glucose was purchased from the National Bureau of Standards (Washington, DC). Tetramethylammonium hydroxide and *N,N*-dimethylacetamide were from EM Science (Cherry Hill, NJ). Pentafluorobenzyl bromide was purchased from Pierce (Rockford, IL). The compound 1-monoolein was purchased from Serdary Research Laboratories (London, Ontario, Canada), and RHC 80267 was obtained from Rorer Central Research (Fort Washington, PA). Other chemicals were obtained either from Sigma or from Fisher Scientific (Pittsburgh, PA). All organic solvents were obtained from Burdick and Jackson (Muskegon, MI). The following products were purchased from Amersham Corp. (Arlington Heights, IL): 1-stearoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycerol (58.3 mCi/mmol); 1,2-di[1-^{14}C]myristoyl-L-3-phosphatidylcholine (108 mCi/mmol); and the liquid scintillation cocktail ACS. Products obtained from New England Nuclear Products (Boston, MA) included [2-^{3}H]glycerol (11.5 Ci/mmol); [*methyl*-^{3}H]choline chloride (80 Ci/mmol), and [*methyl*-^{14}C]phosphorylcholine (50 mCi/mmol).

Isolation and Culture of Islets. Islets were isolated aseptically from male Sprague-Dawley rats, fed ad libitum as described elsewhere (Turk et al., 1984b). In brief, the pancreas was inflated with Hanks' balanced salt solution (supplemented with 0.5% penicillin–streptomycin), excised, and freed from adherent lymphatic, vascular, and adipose tissue. The pancreatic tissue was then digested with collagenase (5 mg/mL at 39 °C for 12.5 min), rinsed with Hanks' solution, and centrifuged on a discontinuous Ficoll gradient (four layers of 27%, 23%, 20.5%, and 11%). Islets were collected from the 11–20.5% interface and washed in CMRL-1066 tissue culture medium (supplemented with 1% penicillin–streptomycin, 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 5.55 mM glucose). This procedure typically yielded 300–400 islets/rat. Isolated islets were then cultured overnight in four petri dishes with 2.5 mL of complete CMRL-1066 at 24 °C under an atmosphere of 95% air/5% CO_2.

Labeling of Islets with [^{3}H]Glycerol. Isolated islets (ca. 3000) were incubated for 48 h in a petri dish containing 2.5 mL of CMRL-1066 tissue culture medium and 50 μCi of [^{3}H]glycerol at 37 °C under an atmosphere of 95% air/5% CO_2. Islets were then washed 3 times in Hepes–Krebs–3 mM glucose medium (25 mM Hepes, pH 7.4, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% fatty acid free bovine serum albumin). The labeled islets were then counted randomly (300/tube) under stereomicroscopic visualization and placed in silanized 16 × 100 mm borosilicate tubes containing Hepes–Krebs medium (3 mM glucose).

Labeling of Islets with [^{3}H]Choline. Isolated islets (ca. 6000) were incubated for 48 h in a petri dish containing 2.5 mL of CMRL-1066 tissue culture medium and 50 μCi of [^{3}H]choline at 37 °C under an atmosphere of 95% air/5% CO_2. Islets were then washed 3 times in Hepes–Krebs–3 mM glucose medium supplemented with 1 mM choline. The labeled islets were then placed into test tubes as described above.

Incubation of Islets. For mass measurement experiments, isolated islets (ca. 12000) were washed 3 times in Hepes–Krebs–3 mM glucose medium, counted (1000/tube) into 16 × 100 mm silanized borosilicate tubes, and preincubated 30 min with shaking in 0.5 mL of Hepes–Krebs–3 mM glucose at 37 °C under an atmosphere of 95% air/5% CO_2. The medium was then removed from each tube and replaced with 0.5 mL of fresh Hepes–Krebs medium supplemented with the appropriate agonist (28 mM glucose or 0.5 mM carbachol) prewarmed to 37 °C. The tubes were then incubated with shaking for 1, 2, 5, 10, or 30 min at 37 °C under an atmo-

sphere of 95% air/5% CO_2. At the end of this period, the medium was removed, and its insulin content was subsequently determined by radioimmunoassay. To the islet pellet was added 0.5 mL of ice-cold methanol. The tubes were then chilled for 15 min in a liquid N_2 bath and stored at −70 °C prior to extraction. For radiochemical experiments, labeled islets were incubated and incubations terminated in a manner similar to that described above. In experiments involving [^{3}H]choline-labeled islets, incubations were terminated with 1 mL of solvent A (chloroform/methanol, 2:1 v/v) which had been previously chilled in a solid CO_2/2-propanol bath.

Extraction of Lipids and TLC Analysis of [^{3}H]Glycerol-Labeled Islets. An internal standard solution consisting of 2000 cpm of 1-stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol and 20 μg of 1-stearoyl-2-arachidonyl-*sn*-glycerol in 0.25 mL of diethyl ether was added shortly after terminating the incubations. Tubes were sonicated 30 min in a water bath (+4 °C) and vortexed. To each tube was added 1.25 mL of solvent B (chloroform/methanol, 1:2 v/v) followed by vortexing (30 s), addition of $CHCl_3$ (0.66 mL), vortexing (30 s), addition of H_2O (0.66 mL), vortexing (1 min), and centrifugation (3 min in a table-top centrifuge). The upper (aqueous) layer was discarded. The lower (organic) layer was transferred to a silanized 3.5-mL Schwartz vial, concentrated to dryness under nitrogen, reconstituted in diethyl ether (0.5 mL), transferred to a silanized 1-mL conical vial, concentrated, and applied to a 20 × 20 cm TLC plate (Analtech silica gel G channeled plate). The plate was developed in solvent C (benzene/diethyl ether/ethanol, 35:10:1 v/v). Standard, unlabeled mono-, di-, and triacylglycerol standards were run as controls and were visualized with I_2 vapor. The spots corresponding to monoacylglycerol, 1,2-diacyl-*sn*-glycerol, and triacylglycerol were scraped into scintillation vials. Diethyl ether (1 mL) and liquid scintillation cocktail (3.5 mL) were added, and vials were counted in a liquid scintillation spectrometer under a double-isotope setting (^{14}C, ^{3}H). Results are expressed as a ratio of the ^{3}H counts (from [^{3}H]glycerol) over the ^{14}C counts (from the internal standard).

Extraction and TLC Analysis of [^{3}H]Choline-Labeled Islets. An internal standard consisting of 2300 cpm of [*methyl*-^{14}C]phosphocholine and 50 μg of unlabeled phosphocholine in 0.05 mL of methanol/water (1:1 v/v) was employed for the recovery of water-soluble choline adducts. A separate internal standard solution consisting of 2200 cpm of 1,2-di[1-^{14}C]-myristoyl-L-3-phosphatidylcholine, 10 μg of 1-stearoyl-2-arachidonyl-L-3-phosphatidylcholine, and 25 μg of 1-oleoyl-2-lyso-L-3-phosphatidylcholine in 0.05 mL of $CHCl_3$/methanol (9:1 v/v) was employed for recovery of lipid choline adducts. Tubes were processed essentially as described above for [^{3}H]glycerol-labeled islets with the following modifications: After sonication, water (0.6 mL) was added, and the tubes were centrifuged (15 min, 1500 rpm). The $CHCl_3$ layer was collected, and $CHCl_3$ (1 mL) was added to the supernatant, followed by vortexing (1 min) and centrifugation. The resultant $CHCl_3$ layer was combined with the first and evaporated under nitrogen. The supernatant containing water-soluble choline adducts was evaporated under nitrogen, reconstituted in 0.1 mL of solvent D (50% ethanol), evaporated, and reconstituted in 0.02 mL of solvent D. The $CHCl_3$ layer containing the lipid choline adducts was evaporated, concentrated, and reconstituted in solvent A (0.1 mL). Water-soluble choline adducts were analyzed on a channeled Whatman LK6-D plate (activated at 80 °C for 30 min) developed in solvent E (methanol/0.9% NaCl/28% NH_4OH, 140:100:10 v/v). Standard, unlabeled choline, phosphocholine, CDP-choline, and glycerophosphocholine were run as controls and visualized with I_2 vapor. The spots eluting at the same R_f as the controls were scraped into scintillation vials, to which 0.5 mL of solvent F (50% methanol) and liquid scintillation cocktail (10 mL) were then added. Lipid choline adducts were analyzed on a channeled Analtech silica gel G plate (heat activated 30 min at 80 °C) developed in solvent G (chloroform/methanol/28% NH_4OH/H_2O, 67.5:52.5:4:11 v/v). Standard, unlabeled phosphatidylcholine and lysophosphatidylcholine were run as controls and visualized with I_2 vapor. The spots eluting at the same R_f as the controls were scraped into scintillation vials, to which methanol (0.5 mL) and liquid scintillation cocktail (10 mL) were then added. Vials were counted in a liquid scintillation spectrometer under a double-isotope setting (^{14}C, ^{3}H) and results expressed as a ratio of the ^{3}H/^{14}C counts as described above.

Extraction of Diacylglycerol. Prior to extraction, tubes for the mass measurement experiments received 0.25 mL of an internal standard solution (100 ng of 1,2-dierucoyl-*sn*-glycerol) prepared from 1,2-dierucoyl-*sn*-glycero-3-phosphocholine with *B. cerus* phospholipase C by methods described elsewhere (Majerus & Prescott, 1982). To each tube was then added solvent B (1.25 mL), followed by sonication (30 min, 4 °C), vortexting, addition of $CHCl_3$ (0.66 mL), addition of H_2O (0.66 mL), and centrifugation (3 min in a table-top centrifuge). The lower (organic) layer was transferred (acid-washed silanized Pasteur pipet) to an acid-washed, silanized 3.5-mL glass vial, concentrated under N_2, and reconstituted in heptane (0.25 mL).

HPLC Analysis of 1,2-Diacyl-sn-glycerol. Extracted samples were analyzed on a μPorasil column (3.9 mm × 30 cm, Waters Associates) with a Waters Resolve silica guard column on a Waters HPLC system consisting of a U6K injector, two Model 510 pumps, an automated gradient controller, and a Model 481 LC spectrophotometer. Solvent was delivered at a rate of 2 mL/min with the following program: solvent H (hexane/2-propanol/acetic acid, 100/1/0.01 v/v) for 13 min, a linear gradient over 5 min to solvent I (hexane/2-propanol/acetic acid, 100/10/0.01 v/v), and then solvent I for 7 min. After each run, the solvent composition was changed from I to H as a linear gradient over 5 min, and solvent H was pumped through the column for 15 min before the next injection. Twice each day, the column was standardized with 1-stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycerol. The retention volumes of standard glycerolipids were as follows: triacylglycerol (5 mL); 1,3-diacyl-*sn*-glycerol (13 mL); 1,2-diacyl-*sn*-glycerol (21 mL); and monoacylglycerol (50 mL). Phospholipids did not elute in this system and were retained on the guard column, which was changed after every 15 runs. Islet-derived 1,2-diacyl-*sn*-glycerol was collected into an acid-washed, silanized 7-mL glass vial, concentrated under N_2, and stored in 0.5 mL of heptane prior to hydrolysis.

Hydrolysis of 1,2-Diacyl-sn-glycerol to Free Fatty Acids. Following evaporation under N_2, to each sample was added 0.4 mL of methanol and 0.1 mL of NaOH (0.2 N). Each tube was incubated 3.5 h at 50 °C under N_2 and adjusted to pH 3.5 (0.1 N HCl). This was followed by addition of CH_2Cl_2 (0.5 mL), vortexing, and centrifugation (1 min, table-top centrifuge). The lower phase was removed, placed in a 1-mL acid-washed, silanized glass vial, concentrated, and reconstituted in CH_2Cl_2 (0.1 mL).

Pentafluorobenzyl Esterification of Free Fatty Acids. The sample was concentrated under N_2, reconstituted in 0.01 mL of solution J (0.8 mL of *N*,*N*-dimethylacetamide, 0.05 mL of tetramethylammonium, and 0.15 mL of methanol) and 0.01

mL of solution K (0.1 mL of pentafluorobenzyl bromide and 0.3 mL of *N,N*-dimethylacetamide), and incubated 15 min at room temperature. The contents were then concentrated to dryness (N_2), and H_2O (0.1 mL) was added. Extraction was performed twice with CH_2Cl_2 (0.2 mL). The organic extract was evaporated under nitrogen, and the sample was reconstituted in heptane (0.05 mL) prior to analysis.

Gas Chromatography–Mass Spectrometry. Gas chromatography (GC) was performed on a Hewlett Packard 5840 gas chromatograph interfaced with a Hewlett Packard 5985B mass spectrometer. A capillary column (Hewlett Packard Ultra-performance capillary column, 8-m length, cross-linked methylsilicone, i.d. 0.31 mm, film thickness 0.17 μm) was operated with a Grob-type injector in the splitless mode with helium as carrier gas (inlet pressure 4 lb/in^2, injector temperature 250 °C). The distal end of the column was inserted directly into the ion source (interface temperature 275 °C). The GC oven temperature was programmed from 85 to 200 °C at a rate of 30 °C/min starting 0.5 min after injection. The mass spectrometer (MS) was operated in the negative ion–chemical ionization (NI–CI) mode with methane as reagent gas (source pressure 1.0 torr). Selected ions were monitored which corresponded to the m/z [M − 181] ions of the fatty acid pentafluorobenzyl ester derivatives. Peak identity was assigned by the mass of the monitored ions and by retention times relative to standard fatty acid pentafluorobenzyl esters, which were determined before each set of analyses. The fatty acid pentafluorobenzyl esters were quantitated relative to erucoylpentafluorobenzyl ester (C22:1 internal standard). Prior to each set of analyses, a standard curve for each fatty acid pentafluorobenzyl ester was generated. Blank samples derived from incubation medium containing no islets were processed in parallel with islet-derived samples in all experiments, and any observed blank signal was subtracted from the signal of islet-derived samples. Background amounts of fatty acids in blank tubes were reduced to 201 ± 25 pmol of palmitate, 186 ± 39 pmol of stearate, 119 ± 13 pmol of oleate, 15 ± 3 pmol of linoleate, and 3 ± 2 pmol of arachidonate by strictly excluding plasticware from all phases of processing, by employing only acid-washed, silanized glassware (including Pasteur pipets), by wearing disposable gloves throughout sample preparation, and by screening solvents and reagents for fatty acid content and employing only those with the minimal obtainable amounts. Since the total fatty acid background was about 500 pmol and the fatty acyl content of islet diacylglycerol was about 1 pmol/islet, at least 1000 islets were employed per condition for mass measurement experiments to achieve an islet signal 2-fold or greater above background.

Insulin Secretion. Isolated islets were randomly counted (20/tube) into 10 × 75 mm borosilicate tubes and preincubated for 30 min at 37 °C with shaking in 0.2 mL of Hepes–Krebs–3 mM glucose media under an atmosphere of 95% air/5% CO_2. The medium was then removed from each tube and replaced with fresh medium (0.2 mL) supplemented with the appropriate agonist. Islets were then incubated with shaking at 37 °C for the appropriate time period. At the end of this period, the supernatant was withdrawn and placed on ice. Appropriate dilutions were then made, and samples were stored at −20 °C prior to determination of their insulin content by radioimmunoassay.

Results

Isolated islets readily incorporated [^{3}H]glycerol into glycerolipids during a 3-h labeling period. Figure 1A illustrates the normal-phase HPLC analysis of radiolabeled neutral lipids

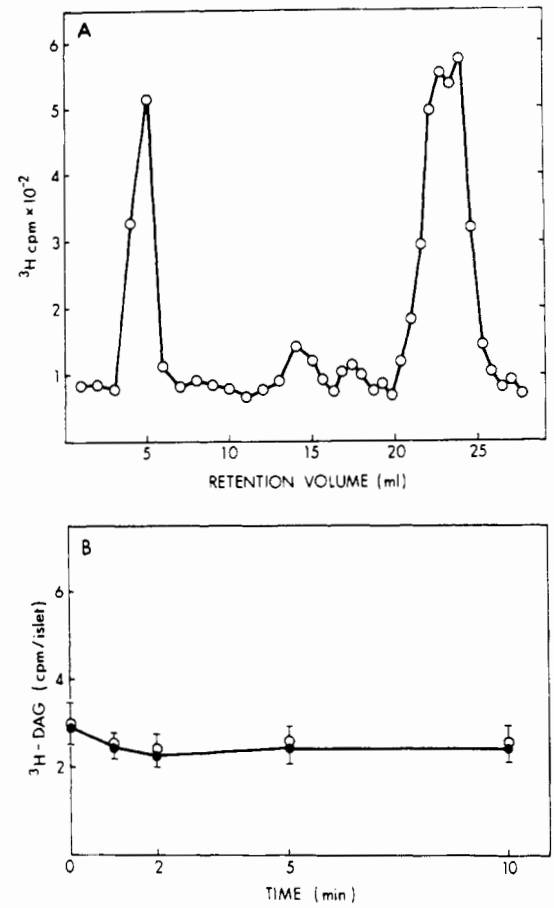

FIGURE 1: Analysis of diacylglycerol from [^{3}H]glycerol-prelabeled islets. (Panel A) Islets were labeled 3 h with [^{3}H]glycerol (50 μCi). Unincorporated radiolabel was removed by extensive washing as described under Experimental Procedures. The islets were then stimulated with 28 mM glucose for 2 min. Neutral lipids were extracted as described under Experimental Procedures. HPLC analysis was performed on a μPorasil analytical column at a flow rate of 2 mL/min with solvent H as the mobile phase. (Panel B) Time course of glucose-induced accumulation of [^{3}H]diacylglycerol in [^{3}H]-glycerol-prelabeled islets. Islets were labeled with [^{3}H]glycerol as in panel A and incubated (300 islets/tube) at 37 °C in Hepes–Krebs medium supplemented with glucose [3 mM (open circles) or 28 mM (closed circles)]. [^{3}H]Diacylglycerol was analyzed by normal-phase HPLC as in panel A. Results are shown as the mean ± SE (n = 7) of radioactivity in diacylglycerol (cpm/islet).

from such islets, including 1,2-diacyl-*sn*-glycerol (retention volume 21–24 mL) and triacylglycerol (retention volume 4 mL). [The diacylglycerol peak contained multiple incompletely resolved components, reflecting molecular heterogeneity in the fatty acyl composition of diacylglycerols as reported by Boccckino et al. (1985).] As illustrated in Figure 1B, D-glucose, at a concentration (28 mM) which maximally stimulates insulin secretion, had no effect on the accumulation of [^{3}H]-diacylglycerol in these [^{3}H]glycerol-labeled islets. This could have reflected (a) a failure of D-glucose to augment islet diacylglycerol accumulation, (b) a failure to label glucose-sensitive pools of a precursor glycerophospholipid, or (c) diacylglycerol production by a mechanism other than hydrolysis of preexisting glycerolipids. To further explore these possibilities, a quantitative approach was used to measure the mass and to determine the fatty acid composition of 1,2-diacyl-*sn*-glycerol in islets. This approach involved addition of 1,2-dierucoyl-*sn*-glycerol (22:1-DAG) as an internal standard. (The fatty acid C22:1 was determined to be less than 1% as abundant as palmitate in rat islet phospholipids.) Endogenous 1,2-diacyl-*sn*-glycerol and the internal standard were then

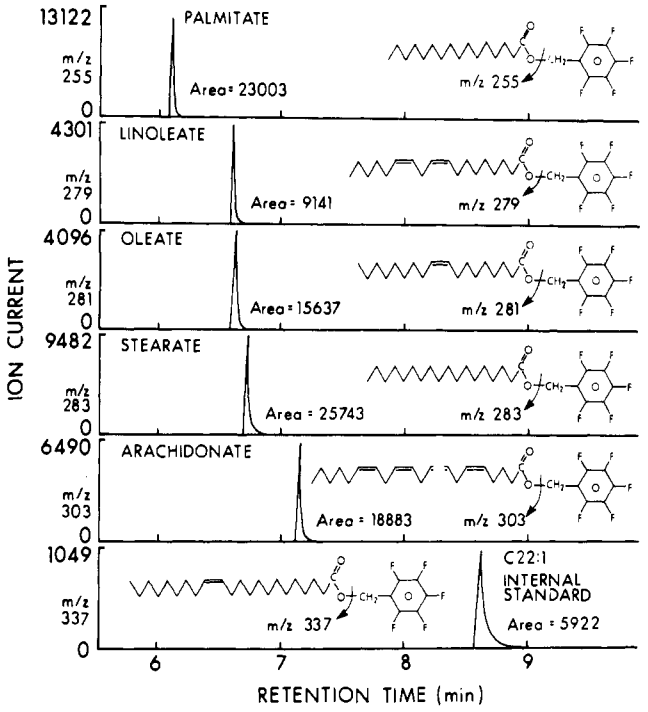

FIGURE 2: Gas chromatographic–negative ion–chemical ionization–mass spectrometric analysis of the fatty acyl content of islet 1,2-diacyl-*sn*-glycerol. Following incubation of 1000 islets/tube, 100 ng of the internal standard (1,2-dierucoyl-*sn*-glycerol) was added to each tube. Neutral lipids were extracted as described under Experimental Procedures. Islet 1,2-diacyl-*sn*-glycerol was purified by normal-phase HPLC and then saponified to its constituent free fatty acids. The free fatty acids were converted to their pentafluorobenzyl (PFBE) esters and reconstituted in heptane. Two microliters of this solution was injected into a Hewlett Packard 5840 gas chromatograph interfaced with a Hewlett Packard 5985B mass spectrometer operated in the negative ion–(methane) chemical ionization mode as described under Experimental Procedures. Selected ions were monitored which corresponded to the molecular weight of the fatty acid PFBE derivatives minus 181, reflecting the loss of the pentafluorobenzyl moiety as illustrated by the structural diagrams of the PFBE fatty acids in the figure.

isolated by normal-phase HPLC and hydrolyzed to their constituent fatty acids. The free fatty acids were then converted to their pentafluorobenzyl esters (PFBE) and analyzed by gas chromatography–negative ion–chemical ionization–mass spectrometry (GC–NI–CI–MS) as illustrated in Figure 2.

The fatty acyl composition of 1,2-diacyl-*sn*-glycerol in islets as determined by this method under basal conditions (3 mM glucose) is illustrated in Figure 3. The predominant fatty acids were stearate (C18:0), arachidonate (C20:4), palmitate (C16:0), oleate (C18:1), and linoleate (C18:2). Trace amounts of myristate (C14:0, 1 fmol/islet), palmitoleate (C16:1, 7 fmol/islet), linoleate (C18:3, 1 fmol/islet), dihomo-γ-linoleate [C20:3(*n*-6), 19 fmol/islet], eicosapentaenoate (C20:5, 1 fmol/islet), adrenate [C22:4(*n*-6), 10 fmol/islet], docosapentaenoate [C22:5(*n*-3), 3 fmol/islet], and docosahexaenoate [C22:6(*n*-3), 10 fmol/islet] were also observed. The total fatty acid mass in islet 1,2-diacyl-*sn*-glycerol under basal conditions was 1013 fmol/islet (range 850–1175 fmol/islet).

The influence of glucose on islet 1,2-diacyl-*sn*-glycerol content was then examined by using the quantitative mass spectrometric methods described above. The results of such experiments are summarized in Figure 4. Under conditions where glucose induced insulin secretion (Figure 4A), glucose also induced accumulation of 1,2-diacyl-*sn*-glycerol by mass (Figure 4B–D). Accumulation of endogenous 1,2-diacyl-*sn*-

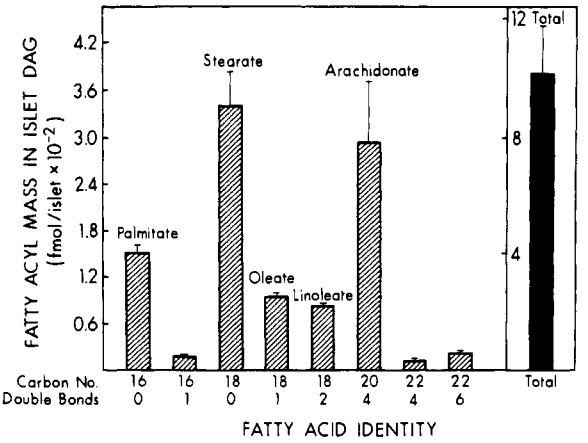

FIGURE 3: Fatty acyl composition of islet 1,2-diacyl-*sn*-glycerol under basal conditions. Isolated islets were cultured overnight at 24 °C in complete CMRL-1066 medium as described under Experimental Procedures. Four thousand islets were counted into each tube and were preincubated for 30 min in Hepes–Krebs medium (25 mM Hepes, pH 7.4, 115 mM NaCl, 24 mM $NaHCO_2$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% fatty acid free bovine serum albumin) supplemented with 3 mM glucose. The medium was then removed and replaced with fresh Hepes–Krebs–3 mM glucose medium, and the islets were incubated for 10 min at 37 °C. The fatty acid composition of islet-derived 1,2-diacyl-*sn*-glycerol was determined by gas chromatographic–negative ion–chemical ionization–mass spectrometric analysis as shown in Figure 2.

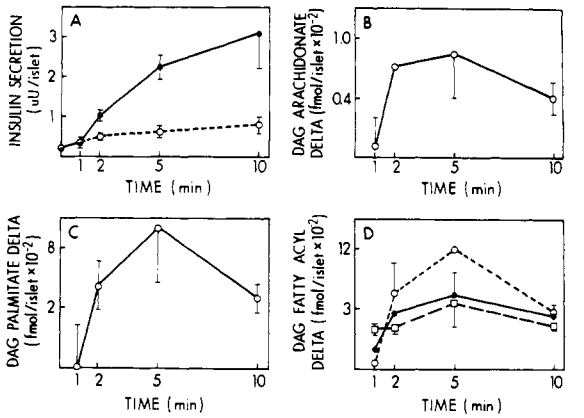

FIGURE 4: Effect of glucose on islet 1,2-diacyl-*sn*-glycerol (DAG) mass and on insulin secretion. Islets were cultured overnight in complete CMRL-1066 medium at 24 °C. The next morning, 1000 islets were counted into each tube. Islets were preincubated 30 min at 37 °C in Hepes–Krebs–3 mM glucose medium. The medium was removed and replaced with fresh medium containing glucose (3 or 28 mM). Islets were then incubated for 1–10 min at 37 °C. The supernatant was removed and assayed for insulin content by radioimmunoassay. The pellet was processed for mass measurement of diacylglycerol and determination of its fatty acid content as shown in panels B, C, and D. (Panel A) Glucose-induced insulin secretion. Results are shown as the mean ± SE of insulin secretion (microunits per islet) from nine experiments: 28 mM glucose (closed circles, solid line); 3 mM glucose (open circles, dashed line). (Panel B) Arachidonate content of islet 1,2-diacyl-*sn*-glycerol. Following incubation, the islet pellet was processed as described under Experimental Procedures, and the fatty acyl composition of 1,2-diacyl-*sn*-glycerol (DAG) was determined as shown in Figure 2. Results are shown as the mean ± SE of the difference between 28 and 3 mM glucose (DELTA) in femtomoles per islet from nine experiments. (Panel C) Palmitate content of islet 1,2-diacyl-*sn*-glycerol. Results are shown as the mean ± SE of the DELTA (femtomoles per islet) from nine experiments. (Panel D) Stearate, oleate, and linoleate content of islet 1,2-diacyl-*sn*-glycerol. Results are shown as the mean ± SE of the DELTA (femtomoles per islet) from nine experiments: C18:0 (closed circles, solid line); C18:1 (open circles, broken line); C18:2 (open squares, dashed line).

glycerol was first observed at 2 min and achieved a maximum at 5 min. The fatty acyl composition of endogenous 1,2-di-

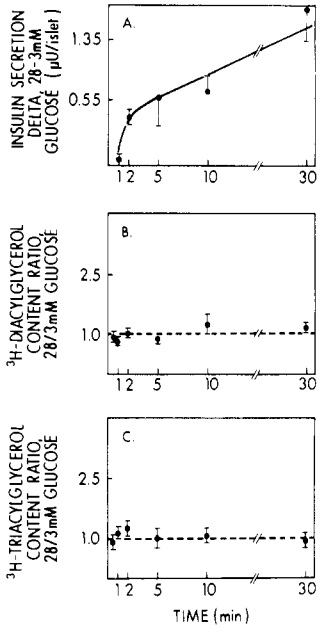

FIGURE 5: Effect of glucose on the accumulation of ^{3}H-labeled neutral lipids and on insulin secretion in islets subjected to long-term prelabeling with [^{3}H]glycerol. Isolated islets were labeled with [^{3}H]-glycerol (50 μCi) in 2.5 mL of complete CMRL-1066 (5.6 mM glucose) tissue culture medium for 48 h at 37 °C. Islets were washed 3 times in Hepes–Krebs–3 mM glucose medium, and then 300 islets were counted into each tube. Islets were preincubated 30 min at 37 °C in Hepes–Krebs–3 mM glucose. The medium was removed and replaced with fresh Hepes–Krebs medium supplemented with 3 or 28 mM glucose. The islets were then incubated for 30 min at 37 °C. The supernatant was removed for measurement of insulin content by radioimmunoassay. The islet pellet was extracted, and neutral lipids were analyzed by TLC as described under Experimental Procedures and as shown in panels B, C, and D. (Panel A) Glucose-induced insulin secretion. Results are shown as the mean ± SE of insulin secretion (microunits per islet) from four experiments expressed as the difference (DELTA) between 28 and 3 mM glucose. (Panel B) Glucose-induced 1,2-[^{3}H]diacyl-*sn*-glycerol (DAG) accumulation. Results are shown as the mean ± SE of islet [^{3}H]DAG content from four experiments expressed as the ratio between 28 mM glucose over 3 mM glucose. Recovery of [^{3}H]DAG was normalized by monitoring the recovery of a [^{14}C]DAG internal standard as described under Experimental Procedures. (Panel C) Glucose influence on islet [^{3}H]triacylglycerol (TAG) content. Results are shown as the mean ± SE of islet [^{3}H]TAG content from four experiments expressed as the ratio between 28 mM glucose over 3 mM glucose.

acyl-*sn*-glycerol accumulating in response to 28 mM glucose was different from that observed under basal conditions (3 mM glucose, Figure 3). After a 5-min incubation with 28 mM glucose, the predominant fatty acyl substituents in 1,2-diacyl-*sn*-glycerol were palmitate (Figure 4C) and oleate (Figure 4D). Smaller amounts of stearate and linoleate (Figure 4D) were also present. Arachidonate was quantitatively much less abundant than other fatty acyl substituents of the diacylglycerol which accumulated in glucose-stimulated islets.

In view of the discrepancy between glucose-induced accumulation of [^{3}H]diacylglycerol in [^{3}H]glycerol-labeled islets (Figure 1B) and glucose-induced accumulation of diacylglycerol by mass (Figure 4), the influence of glucose was reexamined in islets subjected to long-term labeling with [^{3}H]glycerol to achieve more complete labeling of glycerolipid pools. The influence of glucose on islet [^{3}H]diacylglycerol content was evaluated by using [^{14}C]diacylglycerol as an internal standard to correct for any losses during extraction and TLC analysis. Figure 5A illustrates that glucose stimulated insulin secretion in islets subjected to long-term labeling with [^{3}H]glycerol but glucose did not influence the [^{3}H]glycerol content of any neutral glycerolipid, including 1,2-[^{3}H]di-

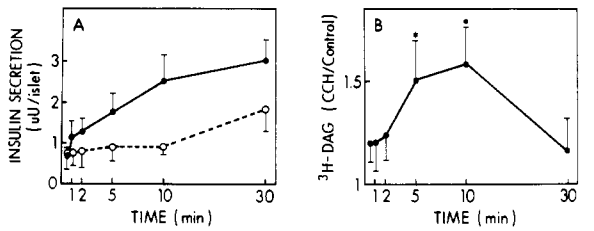

FIGURE 6: Effect of carbachol on the accumulation of 1,2-[^{3}H]diacyl-*sn*-glycerol and on insulin secretion in islets subjected to long-term prelabeling with [^{3}H]glycerol. Isolated islets were prelabeled with [^{3}H]glycerol for 48 h, and unincorporated radiolabel was removed as described in Figure 5. After preincubation (30 min, 37 °C) and removal of the medium (Hepes–Krebs–3 mM glucose), incubation of the [^{3}H]glycerol-prelabeled islets was performed in Hepes–Krebs–3 mM glucose medium supplemented with 0.5 mM carbachol. (Panel A) Carbachol-induced insulin secretion. Results are shown as the mean ± SE of insulin secretion (microunits per islet) from five experiments: 0.5 mM carbachol (closed circles, solid line); control (open circles, dashed line). (Panel B) Carbachol-induced islet 1,2-[^{3}H]-diacyl-*sn*-glycerol (DAG) accumulation. Results are shown as the mean ± SE of [^{3}H]DAG accumulation from five experiments expressed as the ratio of 0.5 mM carbachol over control. Recovery of [^{3}H]DAG was normalized by monitoring the recovery of a [^{14}C]DAG internal standard as described under Experimental Procedures. Asterisks, $p < 0.05$ versus control.

acylglycerol (Figure 5B) or [^{3}H]triacylglycerol (Figure 5C).

In contrast to glucose, the muscarinic agonist carbachol significantly augmented the accumulation of 1,2-[^{3}H]diacyl-*sn*-glycerol in islets prelabeled for 48 h with [^{3}H]glycerol. As shown in Figure 6A, 0.5 mM carbachol significantly stimulated insulin secretion. Carbachol also induced a transient but significant increase in the islet content of 1,2-[^{3}H]diacyl-*sn*-glycerol at 5 and 10 min. There was a return to basal levels after 30 min (Figure 6B). Carbachol also induced a transient accumulation of [^{3}H]monoacylglycerol in the islets (data not shown). Carbachol did not influence the islet content of [^{3}H]triacylglycerol (data not shown).

When examined by the mass spectrometric methods described above, carbachol was also observed to induce accumulation of diacylglycerol by mass in islets, as illustrated in Figure 7. The amplitude and time course of the carbachol-induced increase in endogenous 1,2-diacyl-*sn*-glycerol (Figure 7B) were similar to those seen in the radiochemical experiments (Figure 6B). Both approaches indicated approximately a 50% increase in islet 1,2-diacyl-*sn*-glycerol content after 5 and 10 min of stimulation with carbachol. Analysis of the fatty acid composition of the 1,2-diacyl-*sn*-glycerol which accumulated in carbachol-stimulated islets at 5 min revealed that the material contained stearate, arachidonate, palmitate, and oleate in approximately equal amounts (Figure 7C,D). This fatty acid composition differs from that observed in glucose-stimulated islets (Figure 4) by the greater abundance of arachidonate and stearate in the 1,2-diacyl-*sn*-glycerol in carbachol-stimulated islets. In both cases, the 1,2-diacyl-*sn*-glycerol contains substantial amounts of palmitate and oleate, as does islet phosphatidylcholine (Turk et al., 1986b).

Agonist-induced phosphatidylcholine hydrolysis by phospholipase(s) C to yield diacylglycerol and phosphocholine has now been described in several cell types (Exton, 1988; Rosoff et al., 1988). To evaluate the possibility that phosphatidylcholine hydroysis might contribute to secretagogue-induced diacylglycerol accumulation in islets, long-term labeling experiments were performed with [^{3}H]choline. Prelabeled islets were then stimulated with secretagogues, and water-soluble and lipid choline adducts were analyzed by the methods summarized in Figure 8A,B. Islets readily incorporated

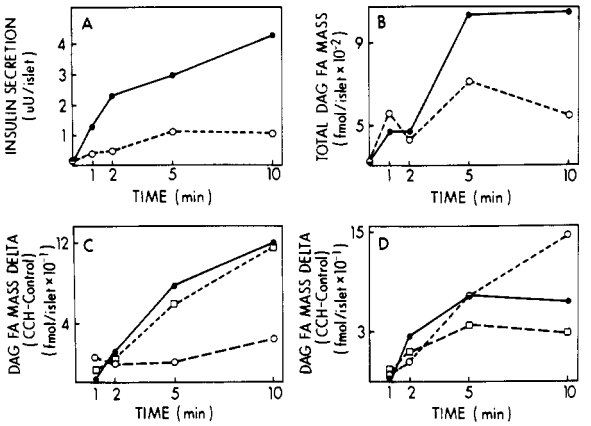

FIGURE 7: Effect of carbachol on islet 1,2-diacyl-*sn*-glycerol (DAG) mass and on insulin secretion. Experiments were performed as described in Figure 4 except that islets (1000/tube) were incubated in Hepes–Krebs–3 mM glucose medium without (control) or with 0.5 mM carbachol. (Panel A) Carbachol-induced insulin secretion. Results are shown as microunits per islet: carbachol (closed circles, solid line); control (open circles, dashed line). (Panel B) Carbachol effect on total fatty acyl (FA) content of islet 1,2-diacyl-*sn*-glycerol (DAG). Results are shown as femtomoles per islet of the total fatty acyl content of DAG. Same symbols as in panel A. (Panel C) Carbachol effect on the myristate, palmitate, and oleate content of islet 1,2-diacyl-*sn*-glycerol (DAG). Results are shown as femtomoles per islet of fatty acid (FA) in DAG expressed as the difference (DELTA) between 0.5 mM carbachol and control: C14:0 (open circles, dashed line); C16:0 (closed circles, solid line); C18:1 (open squares, broken line). (Panel D) Carbachol effect on the stearate, linoleate, and arachidonate content of islet 1,2-diacyl-*sn*-glycerol (DAG): C18:0 (open circles, broken line); C18:2 (open squares, dashed line); C20:4 (closed circles, solid line).

[^{3}H]choline into phosphatidylcholine under these conditions (Figure 8C) and secreted insulin in response to both glucose and carbachol after long-term [^{3}H]choline labeling (Figure 9A). Neither secretagogue, however, induced accumulation of [^{3}H]phosphocholine (Figure 9B) or of free [^{3}H]choline (Figure 9C) in [^{3}H]choline-labeled islets. This indicates that the diacylglycerol that accumulates in islets in response to glucose or carbachol does not arise from hydrolysis of phosphatidylcholine.

The possible participation of secretagogue-induced diacylglycerol accumulation in insulin secretion was investigated by two approaches. The first was to examine the effects of exogenous diacylglycerol on insulin secretion. The second was to examine the influence of inhibiting the metabolism of endogenous diacylglycerol on secretagogue-induced insulin secretion. 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG) is a membrane-permeable diacylglycerol (Kiabuchi et al., 1983). As shown in Figure 10 (left panel), the addition of 100 μM OAG amplified glucose (8 mM)-induced insulin secretion significantly by more than 2-fold. Under basal conditions, OAG also induced insulin secretion in a concentration-dependent manner (Figure 10, right panel). Exogenous diacylglycerol therefore clearly augments insulin secretion from isolated islets.

The principal routes of metabolism of endogenous diacylglycerol are hydrolysis by diglyceride lipase or conversion to phosphatidic acid by diglyceride kinase. The former process is inhibited by the agent RHC 80267, and the latter process is inhibited by the agent 1-monooleoyl-*sn*-glycerol (MOG) (Bishop & Bell, 1986). As illustrated in Figure 11, the combination of these two agents increased the mass of islet diacylglycerol under basal conditions and after stimulation with carbachol. The combination of the two agents also resulted in a doubling of insulin secretion under basal conditions (Table I). Carbachol-induced insulin secretion was 27% higher in the presence than in the absence of the combination of the two compounds, but glucose-induced secretion was strongly suppressed (Table I). This latter effect is attributable entirely to the diacylglycerol lipase inhibitor RHC 80267. The diacylglycerol kinase inhibitor MOG alone augmented insulin secretion induced by either glucose or carbachol by more than 2-fold (Table I).

Discussion

This study demonstrates that under resting conditions isolated pancreatic islets contain measurable amounts of endogenous 1,2-diacyl-*sn*-glycerol and that the principal fatty

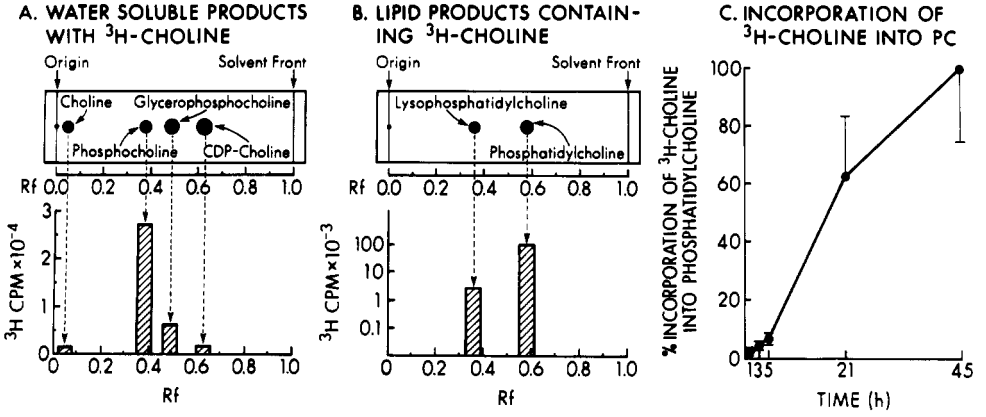

FIGURE 8: Islet [^{3}H]choline labeling and analysis of lipid and water-soluble products. Isolated islets were labeled with [^{3}H]choline (50 μCi) in 2.5 mL of complete CMRL-1066 (5.56 mM glucose) tissue culture medium for 1–48 h at 37 °C. At the end of the labeling period, the islets were washed 3 times in Hepes–Krebs–3 mM glucose medium supplemented with 1 mM choline to remove unincorporated radiolabel. Three hundred islets were then counted into each tube and were preincubated 30 min at 37 °C in Hepes–Krebs–3 mM glucose. The medium was removed and replaced with fresh medium supplemented with the appropriate agonist. The islets were then incubated for 0.5–30 min at 37 °C. The supernatant was removed, and its insulin content was determined by radioimmunoassay. The islet pellet was extracted as described under Experimental Procedures with $CHCl_3/CH_3OH$ after addition of an internal standard solution containing 2300 cpm of [*methyl*-^{14}C]-phosphocholine, 50 μg of phosphocholine, 2300 cpm of 1,2-di[1-^{14}C]myristoyl-L-3-phosphatidylcholine, 10 μg of 1-stearoyl-2-arachidonyl-L-3-phosphatidylcholine, and 25 μg of oleoyl-L-lysophosphatidylcholine. (Panel A) TLC analysis of water-soluble choline adducts. Analysis was performed on a channeled Whatman LK6-D plate (preactivated 30 min at 80 °C) which was developed in CH_3OH/0.9% NaCl/28% NH_4OH (140:100:10 v/v). (Panel B) TLC analysis of lipid choline adducts. Analysis was performed on a channeled Analtech silica gel G plate (preactivated 30 min at 80 °C) which was developed in $CHCl_3/CH_3OH$/28% NH_4OH/H_2O (67.5:52.5:4:11 v/v). (Panel C) Time course of [^{3}H]choline incorporation into islet phosphatidylcholine. Results are shown as the mean ± SE of [^{3}H]choline incorporated into phosphatidylcholine expressed as a percentage of the incorporation observed at 45 h.

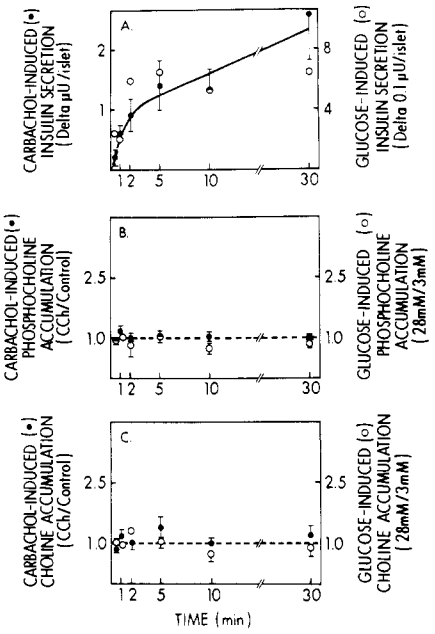

FIGURE 9: Secretagogue effects on [^{3}H]choline-prelabeled islets. Islets were labeled with [^{3}H]choline for 48 h as described in Figure 8 and were incubated under the conditions described below. Water-soluble [^{3}H]choline adducts were analyzed as described in Figure 8. (Panel A) Insulin secretion. Results are shown as the mean ± SE of carbachol-induced (closed circles) or glucose-induced (open circles) insulin secretion expressed as the ratio of stimulated over control from four experiments. (Panel B) Secretagogue-induced [^{3}H]phosphocholine accumulation. Results are shown as the mean ± SE of carbachol-induced (closed circles) or glucose-induced (open circles) accumulation of [^{3}H]phosphocholine expressed as the ratio of stimulated over control from four experiments. Recovery of [^{3}H]phosphocholine was monitored with a [^{14}C]phosphocholine internal standard as described under Experimental Procedures. (Panel C) Secretagogue-induced [^{3}H]-choline accumulation. Results are shown as the mean ± SE of carbachol-induced (closed circles) or glucose-induced (open circles) [^{3}H]choline accumulation expressed as the ratio of stimulated over control from four experiments.

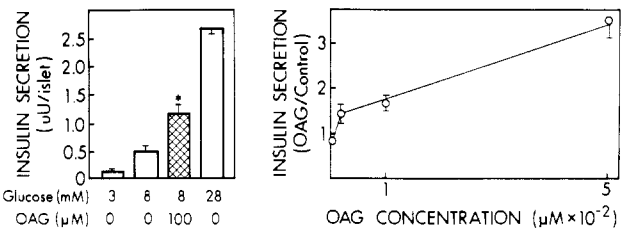

FIGURE 10: Effect of exogenous diacylglycerol on insulin secretion by isolated islets. (Left panel) Islets (20/tube) were preincubated 30 min in Hepes–Krebs–3 mM glucose medium in the absence of bovine serum albumin at 37 °C. The medium was removed and replaced with fresh medium containing glucose (3, 8, or 28 mM) and oleoylacetylglycerol (OAG) (0 or 100 μM). The islets were incubated for 30 min at 37 °C. The supernatant was removed and assayed for insulin content by radioimmunoassay. Results are shown as the mean ± SE of insulin secretion (microunits per islet) from three experiments. Asterisk, $p < 0.05$ versus control. (Right panel) Concentration dependence of insulin secretion induced by OAG under basal conditions (5.6 mM glucose). Results are expressed as the ratio of OAG over control.

acyl constituents of this material are arachidonate and stearate. These are also the principal fatty acyl constituents of phosphoinositides in most tissues (Holub & Kuskis, 1978), including islets (Turk et al., 1986b). Since resting islets contain substantial amounts of inositol phosphates by mass (Turk et al., 1987), phosphoinositide hydrolysis may be an important contributor to the basal 1,2-diacyl-*sn*-glycerol content of islets. Islets stimulated with glucose in concentrations sufficient to induce insulin secretion accumulate increased amounts of

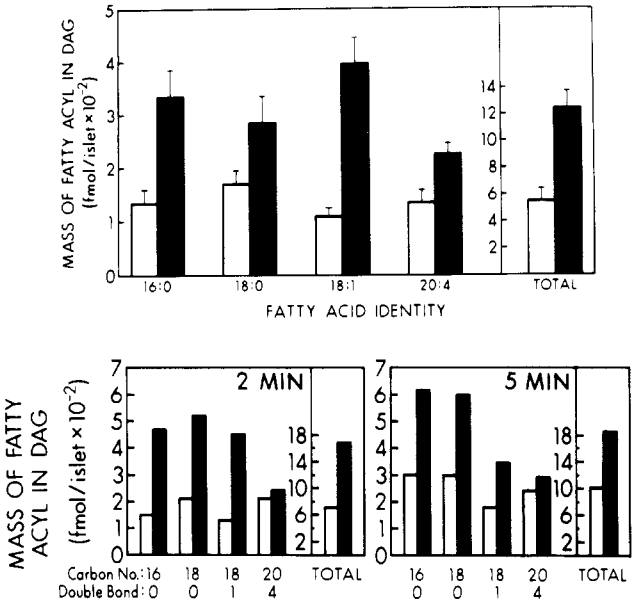

FIGURE 11: Effect of inhibitors of diacylglycerol (DAG) metabolism on carbachol-induced accumulation of endogenous DAG in islets. Experiments were performed as in Figure 4 except that islets (1000/tube) were preincubated 30 min at 37 °C in Hepes–Krebs–3 mM glucose medium supplemented with the following inhibitors of DAG metabolism: 100 μM 1-monoolein and 100 μM RHC 80267. The medium was then removed, and the islets were incubated in fresh medium containing the inhibitors and 0.5 mM carbachol. (Top panel) Effect of inhibitors of DAG metabolism on islet resting DAG mass and fatty acid composition. Results are shown as the mean ± SE (n = 12–14) of the fatty acyl mass in 1,2-diacyl-*sn*-glycerol (femtomoles per islet) in islets incubated without (open bars) or with (solid bars) inhibitors of DAG metabolism. (Bottom panels) Effect of carbachol on islet DAG mass and fatty acid composition in the presence of inhibitors of DAG metabolism. Results are shown as the mean ± SE of the fatty acyl mass in 1,2-diacyl-*sn*-glycerol (femtomoles per islet) for islets incubated with carbachol (0.5 mM) alone (open bars, n = 2) or with carbachol (0.5 mM) and inhibitors of DAG metabolism (solid bars, n = 3).

Table I: Effect of 1-Monoolein and RHC 80267 on Glucose- and Carbachol-Induced Insulin Secretion in Isolated Islets of Langerhans[a]

condition	insulin secretion (microunits islet^{-1} min^{-1}; mean ± SE) 3 mM glucose	28 mM glucose	3 mM glucose + 0.5 mM carbachol
control	0.12 ± 0.02	1.36 ± 0.19	0.22 ± 0.03
100 μM 1-monoolein	0.25 ± 0.04[b]	2.79 ± 0.17[d]	0.48 ± 0.05[c]
100 μM RHC 80267	0.12 ± 0.02	0.29 ± 0.04[d]	0.29 ± 0.07
100 μM 1-monoolein + 100 μM RHC 80267	0.24 ± 0.06[b]	0.28 ± 0.05[d]	0.28 ± 0.05

[a] Isolated islets were cultured overnight in complete CMRL-1066 medium at 24 °C under an atmosphere of 95% air/5% CO_2. The next day, islets were randomly counted (20/tube). Islets were preincubated 30 min at 37 °C with shaking in 0.2 mL of Hepes–Krebs–3 mM glucose medium containing 0.2% DMSO and 0.2% ethanol (control), 100 μM 1-monoolein in 0.2% ethanol, 100 μM RHC 80267 in 0.2% DMSO, or 100 μM 1-monoolein and 100 μM RHC 80267. The medium was then removed from each tube and replaced with 0.1 mL of fresh medium supplemented with 28 mM glucose or 0.5 mM carbachol. Islets were then further incubated 10 min with shaking at 37 °C. The supernatant was removed and its insulin content assayed by radioimmunoassay. Results are expressed as the mean ± SE of insulin secretion (μU/islet/min) from three experiments each performed in quadruplicate. [b] $p < 0.05$. [c] $p < 0.01$. [d] $p < 0.001$ versus control.

1,2-diacyl-*sn*-glycerol by mass. The 1,2-diacyl-*sn*-glycerol that accumulates in glucose-stimulated islets, however, is rich in palmitate and oleate and contains relatively small amounts

of arachidonate. This suggests that this material does not arise primarily from phosphoinositide hydrolysis.

Palmitate and oleate are the most abundant saturated and unsaturated fatty acyl substituents, respectively, in islet phosphatidylcholine (Turk et al., 1986b). Diacylglycerols rich in these fatty acids also accumulate in vasopressin-stimulated hepatocytes (Bocckino et al., 1985; Irving & Exton, 1987; Exton, 1988) and interleukin-1-stimulated lymphocytes (Rosoff et al., 1988). This material arises from phospholipase C catalyzed hydrolysis of phosphatidylcholine in those cases. The activity of such an enzyme in these systems is reflected by the simultaneous generation of diacylglycerol and phosphocholine. A related mechanism of generating diacylglycerol from phosphatidylcholine involves the sequential actions of phospholipase D and phosphatidate phosphohydrolase (Bocckino et al., 1987). This is reflected by agonist-induced generation of free choline which precedes the appearance of diacylglycerol. Islets subjected to long-term labeling with [^{3}H]choline readily incorporate this material into phosphatidylcholine. Islets so labeled secrete insulin in response to glucose but do not accumulate increased amounts of either phosphocholine or free choline in response to glucose. This indicates that the 1,2-diacyl-*sn*-glycerol which accumulates in islets under these conditions does not arise from hydrolysis of phosphatidylcholine. We have not examined the possibility that glucose might induce accumulation of phosphoserine or of phosphoethanolamine in islets, but observations discussed below with [^{3}H]glycerol-prelabeled islets suggest that the diacylglycerol which accumulates in glucose-stimulated islets does not arise from hydrolysis of any preexisting glycerolipid, including phosphatidylserine or phosphatidylethanolamine.

Triglyceride hydrolysis contributes to diacylglycerol accumulation in salivary acinar cells stimulated with β-adrenergic agonists (Solung et al., 1987) and in pancreatic exocrine cells stimulated with muscarinic agonists (Banschbach et al., 1981). Islets subjected to long-term labeling with [^{3}H]glycerol readily incorporate radiolabel into triacylglycerol and secrete insulin in response to glucose. Under these conditions, however, glucose induces neither a decline in the ^{3}H content of islet triacylglycerol nor an increase in the ^{3}H content of islet 1,2-diacyl-*sn*-glycerol. This indicates that triglyceride hydrolysis is not an important source of the 1,2-diacyl-*sn*-glycerol that accumulates in glucose-stimulated islets.

The discrepancy between the mass measurements and [^{3}H]glycerol labeling of 1,2-diacyl-*sn*-gycerol in glucose-stimulated islets indicates that the diacylglycerol which accumulates under these conditions does not arise from hydrolysis of preexisting glycerolipids. This material must rather be synthesized de novo and derive its glycerol moiety from the metabolism of glucose. Glucose carbon is known to be incorporated into islet diacylglycerol (Vara & Tamarit-Rodriguez, 1986) by a process which involves metabolism of glucose via the glycolytic pathway to dihydroxyacetone phosphate. This intermediate is then acylated, reduced to lysophosphatidic acid, acylated to phosphatidic acid, and hydrolyzed by phosphatidate phosphohydrolase to yield diacylglycerol (Dunlop & Larkins, 1985a,b).

By contrast, islets subjected to long-term labeling with [^{3}H]glycerol and then stimulated with the muscarinic agonist carbachol do accumulate increased amounts of ^{3}H label in 1,2-diacyl-*sn*-glycerol and exhibit a comparable increment in 1,2-diacyl-*sn*-glycerol fatty acyl mass. This indicates that hydrolysis of preexisting glycerolipids is an important contributor to 1,2-diacyl-*sn*-glycerol accumulation in carbachol-stimulated islets. Experiments similar to those described above indicate that carbachol does not stimulate hydrolysis of islet phosphatidylcholine or of triacylglycerol. The 1,2-diacyl-*sn*-glycerol which accumulates in carbachol-stimulated islets contains much more arachidonate than that in glucose-stimulated islets and contains stearate in amounts comparable to arachidonate. The fatty acid composition and the known effect of carbachol to induce accumulation of inositol phosphates in islets (Biden et al., 1987; Wolf et al., 1988a,b) suggest that phosphoinositide hydrolysis contributes to 1,2-diacyl-*sn*-glycerol accumulation in carbachol-stimulated islets. The fuel secretagogue D-glucose and the muscarinic receptor agonist carbachol therefore induce 1,2-diacyl-*sn*-glycerol accumulation in islets by at least two distinct mechanisms.

That other, as yet undefined, mechanisms may contribute to islet diacylglycerol generation is suggested by the progressive rise in the 1,2-diacyl-*sn*-glycerol content of palmitate as a function of time after stimulation with carbachol. It is at present uncertain whether this reflects hydrolysis of glycerolipids other than those considered above, an effect of carbachol to stimulate de novo glycerolipid synthesis, or some other phenomenon. While this work was in progress, Peter-Riesch et al. (1988) reported the results of a study employing somewhat different methods in which secretagogue-induced diacylglycerol accumulation in islets was also examined. These workers also observed substantial amounts of palmitate in the diacylglycerol which accumulates in carbachol-stimulated islets. This group and others (Vara & Tamarit-Rodriguez, 1986) have observed increased incorporation of [^{14}C]glucose into the diacylglycerol of glucose-stimulated islets, which is compatible with the hypothesis that glucose enhances de novo synthesis of islet diacylglycerol.

It is possible that the metabolism of glucose to the triose phosphate dihydroxyacetone phosphate and then to diacylglycerol as outlined above relates to a long appreciated enigma in the properties of carbohydrates as islet fuels and insulin secretagogues (Ashcroft, 1980; Meglasson & Matschinsky, 1986). Carbohydrates that induce insulin secretion include glucose, mannose, *N*-acetylglucosamine, dihydroxyacetone, and glyceraldehyde. All of these compounds are converted to triose phosphates by islets (Ashcroft, 1980). By contrast, lactate and pyruvate, although readily oxidized in the mitochondria of islets, cannot be converted by islets to triose phosphates and do not induce insulin secretion (Ashcroft, 1980; Meglasson & Matschinsky, 1986). This has led to the hypothesis that a metabolic signal involved in insulin secretion is generated from the glycolytic pathway at the level of the triose phosphates (Ashcroft, 1980; Meglasson & Matschinsky, 1986). It may be that diacylglycerol represents such a signal.

Wollheim et al. (1988) have recently reported that glyceraldehyde induces accumulation of diacylglycerol derived from de novo synthesis in clonal insulin-secreting RIN 5F cells and that exogenous diacylglycerol and tumor-promoting phorbol esters induce closure of an ATP-sensitive potassium channel in these cells. The closure of such a channel in islets is thought to be an early event in the response to glucose and to lead to membrane hyperpolarization and Ca^{2+} influx through voltage-sensitive channels (Cook & Hales, 1984; Ashcroft et al., 1984; Misler et al., 1986; Cook et al., 1988). Whether glucose-induced diacylglycerol accumulation participates in these events in islets remains to be determined.

The possibility that the endogenous diacylglycerol which accumulates in secretagogue-stimulated islets participates in insulin secretion is supported by our and others' (Malaisse et al., 1985) observations that exogenous diacylglycerols induce insulin secretion. Pharmacologic agents which interfere with

the metabolism of diacylglycerol and therefore augment its accumulation in response to secretagogues have mixed effects on insulin secretion. The amplification of secretagogue-induced insulin secretion by the diacylglycerol kinase inhibitor MOG (Bishop & Bell, 1986) is consistent with a prosecretory role of endogenous diacylglycerol. The inhibition of insulin secretion by the diacylglycerol lipase inhibitor RHC 80267 is similar to the impact of the compound on carbachol-induced secretion in pancreatic exocrine tissue (Sekar & Hokin, 1987). This may reflect nonspecific effects of the agent or a role for diacylglycerol hydrolysis products in the secretory process (Metz, 1988; Metz et al., 1987).

In summary, the insulin secretagogues D-glucose and carbachol both induce accumulation of 1,2-diacyl-*sn*-glycerol in isolated pancreatic islets by different mechanisms. Accumulation of 1,2-diacyl-*sn*-glycerol in response to glucose appears to reflect de novo synthesis rather than hydrolysis of preexisting glycerolipids. The conversion of the glucose carbon skeleton to the glycerol backbone of 1,2-diacyl-*sn*-glycerol is known to involve a triose phosphate as an intermediate. It is therefore possible that 1,2-diacyl-*sn*-glycerol represents the metabolic signal postulated to arise from glycolysis which accounts for the discrepancy between the fuel and secretagogue properties of glucose and glyceraldehyde on the one hand and lactate and pyruvate on the other (Ashcroft, 1980; Meglasson & Matschinsky, 1986).

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Registry No. Insulin, 9004-10-8; carbachol, 51-83-2; D-glucose, 50-99-7; arachidonic acid, 506-32-1; stearic acid, 57-11-4; palmitic acid, 57-10-3; oleic acid, 112-80-1.

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Conformation of Recombinant Desulfatohirudin in Aqueous Solution Determined by Nuclear Magnetic Resonance†

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ABSTRACT: The three-dimensional structure of recombinant desulfatohirudin in aqueous solution was determined by ^{1}H nuclear magnetic resonance at 600 MHz and distance geometry calculations with the program DISMAN. The input for the structure calculations was prepared on the basis of complete sequence-specific resonance assignments at pH 4.5 and 22 °C and consisted of 425 distance constraints from nuclear Overhauser enhancements and 159 supplementary constraints from spin–spin coupling constants and from the identification of intramolecular hydrogen bonds. Residues 3–30 and 37–48 form a molecular core with two antiparallel β-sheets and several well-defined turns. The three disulfide bonds 6–14, 16–28, and 22–39 were identified by NMR. In contrast to this well-defined molecular core, with an average root mean square distance for the polypeptide backbone of 0.8 Å for a group of nine DISMAN solutions, no preferred conformation was found for the C-terminal segment 49–65, and a loop consisting of residues 31–36 is not uniquely constrained by the NMR data either. These structural properties of recombinant desulfatohirudin coincide closely with the previously described solution conformation of natural hirudin, but the presence of localized differences is indicated by chemical shift differences for residues Asp 5, Ser 9, Leu 15, Asp 53, Gly 54, and Asp 55.

Hirudin is a small protein of 65 amino acid residues, which was first isolated from the salivary glands of the leech *Hirud medicinalis* (Markwardt, 1970). Hirudin acts as a specific inhibitor of α-thrombin. Strong and exclusive affinity for α-thrombin, low antigenicity, and rapid clearance from the blood make it attractive for medical applications (Markwardt et al., 1982). While its fundamental functional mechanisms were extensively investigated (Chang, 1983; Stone & Hofsteenge, 1986), insufficient amounts of homogeneous hirudin preparations could be obtained from natural sources to warrant practical medical applications. With recombinant methods large amounts of the protein can now be produced (Meyhack et al., 1987; Grossenbacher et al., 1987), which also renews interest in the structure–function correlations of this protein (Braun et al., 1988).

Natural hirudin contains a sulfated tyrosine residue in position 63 (Badgy et al., 1976; Dodt et al., 1984). The recombinant protein lacks this posttranscriptional modification and contains normal tyrosine in position 63. At the same time its inhibitory activity toward α-thrombin is 10 times weaker than that of native hirudin. However, the reduced activity of the recombinant protein cannot at present be simply correlated with the absence of sulfated Tyr 63, since neither the location of the disulfide bridges in desulfatohirudin nor its three-dimensional structure is known. Similar three-dimensional structures for the two proteins were indicated by the observation that the activity of recombinant hirudin corresponds approximately to that of chemically desulfated hirudin (Braun et al., 1988). To establish more direct correlations between the reduced activity of the recombinant desulfatohirudin and its structure, this paper describes the determination of the three-dimensional desulfatohirudin structure in solution by NMR.[1] This structure is then compared with the previously reported solution conformation of natural hirudin (Clore et al., 1987; Sukumaran et al., 1987).

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[1] Abbreviations: NMR, nuclear magnetic resonance; 2D, two dimensional; 2QF-COSY, two-quantum-filtered homonuclear correlated spectroscopy; E-COSY, two-dimensional exclusive correlation spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; TOCSY, two-dimensional total correlation spectroscopy; TPPI, time-proportional phase incrementation; RELAYED-COSY, two-dimensional relayed coherence-transfer spectroscopy; TSP, 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate, sodium salt; RMSD, root mean square difference.

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