Diacylglycerol Hydrolysis to Arachidonic Acid Is Necessary for Insulin Secretion from Isolated Pancreatic Islets: Sequential Actions of Diacylglycerol and Monoacylglycerol Lipases†

Robert J. Konrad, Christopher D. Major, and Bryan A. Wolf*

Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

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ABSTRACT: Arachidonic acid has been implicated as a second messenger in insulin secretion on the basis of (1) mobilization of intracellular Ca^{2+} from the endoplasmic reticulum of islets and (2) amplification of voltage-dependent Ca^{2+} entry. The insulin secretagogues D-glucose and the muscarinic agonist carbachol both increase unesterified arachidonic acid accumulation in isolated islets. We now show that diacylglycerol, a product of phospholipase C action, is a major source of free arachidonic acid in islets. Diacylglycerol hydrolysis in islets occurs through a two-step process. In the first step, the *sn*-1 bond of 1-stearoyl-2-arachidonyl-*sn*-glycerol is hydrolyzed by a diacylglycerol lipase, giving rise to 2-arachidonyl-*sn*-glycerol. Next, the *sn*-2 bond of 2-arachidonyl-*sn*-glycerol is hydrolyzed by a monoacylglycerol lipase, which is the rate-limiting step, releasing unesterified arachidonic acid. Both diacylglycerol lipase and monoacylglycerol lipase are highly enriched in the plasma membrane of β-cells. Diacylglycerol lipase activity in islet homogenates is selectively inhibited in a dose-dependent manner by the compound RHC-80267, a specific diacylglycerol lipase inhibitor. RHC-80267 inhibits glucose- and carbachol-induced insulin release from intact islets in a dose-dependent manner that parallels its inhibition of diacylglycerol lipase activity. Importantly, RHC-80267, at concentrations that almost completely inhibit diacylglycerol lipase activity and glucose- and carbachol-induced insulin secretion by islets, markedly inhibits glucose- and carbachol-induced increases in islet arachidonic acid levels, as measured by gas chromatography with electron-capture detection of its pentafluorobenzyl esters. RHC-80267 did not significantly affect islet glucose oxidation, phospholipase C, monoacylglycerol lipase, or phospholipase A_2. Since glucose and carbachol are known to stimulate phospholipase C, our observations indicate that diacylglycerol is an important source of arachidonic acid and other free fatty acids in islets. Furthermore, production of arachidonic acid from the hydrolysis of diacylglycerol is essential for glucose- and carbachol-induced insulin secretion.

Insulin secretion from the islets of Langerhans can be stimulated by different types of secretagogues (Ashcroft, 1980). D-Glucose, a fuel secretagogue, is the major physiological stimulus (Hedeskov, 1980; Malaisse et al., 1979). Another class of secretagogues consists of agonists that bind to plasma membrane receptors of the β-cell (Zawalich & Rasmussen, 1990). A member of this class, the muscarinic agonist carbachol has been widely used to probe the vagal component of insulin secretion (Prentki & Matschinsky, 1987; Turk et al., 1987; Garcia et al., 1988). In the case of fuel secretagogues, the mechanism by which insulin release is stimulated is not completely elucidated, although glucose oxidation is essential (Matschinsky, 1990; Meglasson & Matschinsky, 1986; Malaisse et al., 1979; Wollheim & Sharp, 1981). Glucokinase is believed to act as a glucose sensor, with the phosphorylation of glucose to glucose 6-phosphate serving as the rate-limiting step in glucose oxidation (Matschinsky et al., 1993). While the inhibition of glucose oxidation inhibits insulin release, the details of the mechanism coupling glucose oxidation to insulin secretion are less clear. It is currently believed that oxidation of fuel secretagogues increases intracellular levels of ATP (Rajan et al., 1990), although this view has been challenged by some groups (MacDonald, 1990). An increased ATP/ADP ratio is believed to close K^+_{ATP} channels at the plasma membrane, resulting in decreased K^+ efflux and subsequent depolarization of the β-cell (Misler et al., 1986; Dunne & Petersen, 1991; Cook et al., 1988). Depolarization then activates voltage-dependent Ca^{2+} channels, causing an influx of extracellular Ca^{2+} into the β-cell and an increase in intracellular Ca^{2+} levels (Wollheim & Sharp, 1981; Prentki & Matschinsky, 1987). Increased intracellular Ca^{2+} activates protein kinases, such as the Ca^{2+}- and calmodulin-dependent protein kinase, resulting in insulin exocytosis (Turk et al., 1987; Colca et al., 1985; Easom et al., 1990; Wollheim & Regazzi, 1990; Wenham et al., 1994).

Glucose, like carbachol, stimulates the enzyme phospholipase C in islets, as evidenced by increases in *myo*-inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$)[1] (Wolf et al., 1988b, 1989; Turk et al., 1986; Peter-Riesch et al., 1988; Biden et al.,

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* Author to whom correspondence should be addressed: Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 217 John Morgan, Philadelphia, PA 19104-6082. Telephone: (215) 898-0025. FAX: (215) 573-2266.

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1987). Ins(1,4,5)P_3 stimulates the release of Ca^{2+} from the endoplasmic reticulum of the β-cell (Prentki et al., 1984; Joseph et al., 1984; Wolf et al., 1985, 1986, 1988a). The other product of phospholipase C stimulation is 1,2-diacyl-*sn*-glycerol. Interestingly, diacylglycerol accumulation is seen after the stimulation of islets with carbachol, but not with glucose (Wolf et al., 1989, 1990; Regazzi et al., 1990; Peter-Riesch et al., 1988). Diacylglycerol is an endogenous activator of protein kinase C (Ganesan et al., 1990; Easom et al., 1990). Protein kinase C activation is observed in carbachol-induced stimulation of islets, but most groups have failed to demonstrate glucose-induced stimulation of protein kinase C (Wang et al., 1993; Ganesan et al., 1990; Calle et al., 1992; Easom et al., 1990; Wollheim & Regazzi, 1990). This disparity has caused controversy over a possible role for diacylglycerol in insulin secretion (MacDonald, 1990; Wollheim & Regazzi, 1990).

The possibility that diacylglycerol may serve as a source of free fatty acids is consistent with the fact that both glucose and carbachol can increase levels of unesterified fatty acids, such as arachidonic acid in islets (Wolf et al., 1986, 1987). Unesterified arachidonic acid mobilizes intracellular Ca^{2+} from the endoplasmic reticulum of the β-cell and, under certain conditions, stimulates insulin release (Wolf et al., 1986, 1987; Wolf et al., 1987). Recently, arachidonic acid has been implicated in the regulation of voltage-dependent Ca^{2+} channels of the β-cell. Arachidonic acid, at the concentrations present in islets, facilitates voltage-dependent Ca^{2+} entry into the β-cell (Wolf et al., 1991; Turk et al., 1993). These observations make arachidonic acid an attractive candidate to be part of a signal transduction mechanism for insulin secretion (Turk et al., 1993).

Two major cellular pathways may account for glucose- and carbachol-induced increases in arachidonic acid: (1) Phospholipase A_2 can directly hydrolyze arachidonic acid from the *sn*-2 position of phospholipid (Konrad et al., 1992a; Jolly et al., 1993; Gross et al., 1993; Ramanadham et al., 1993), and (2) stimulation of phospholipase C can generate diacylglycerol (Wolf et al., 1989, 1990; Regazzi et al., 1990; Peter-Riesch et al., 1988). The relative contribution of either pathway to the total mass accumulation of arachidonic acid and other fatty acids in islets stimulated with glucose and carbachol is unknown.

We now show that diacylglycerol hydrolysis in islets occurs through a two-step sequential pathway. In the first step, the *sn*-1 bond of 1-stearoyl-2-arachidonyl-*sn*-diacylglycerol is hydrolyzed by a diacylglycerol lipase, giving rise to 2-arachidonyl-*sn*-glycerol. Next, the *sn*-2 bond of 2-arachidonyl-*sn*-glycerol is hydrolyzed by a monoacylglycerol lipase, producing unesterified arachidonic acid. Both of these enzymes appear to be localized to the plasma membrane of β-cells, the site of diacylglycerol production. Importantly, selective inhibition of islet diacylglycerol lipase by RHC-80267 [1,6-bis[[[(cyclohexylideneamino)oxy]carbonyl]amino]-hexane] markedly inhibits both glucose- and carbachol-induced increases in arachidonic acid. Inhibition of diacylglycerol lipase also inhibits glucose- and carbachol-induced insulin release, suggesting that diacylglycerol hydrolysis and the subsequent generation of arachidonic acid play an important role in insulin secretion.

[1] Abbreviations: Ins(1,4,5)P_3, *myo*-inositol 1,4,5-trisphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino]ethanesulfonic acid; PGE_2, prostaglandin E_2; MARCKS, myristoylated alanine-rich C kinase substrate.

EXPERIMENTAL PROCEDURES

Materials

Male virus-free Sprague−Dawley rats (200−250 g) were purchased from Charles-River (Wilmington, MA). Collagenase P (lot 20-12/4-92) and *Rhizopus arrhizus* lipase were purchased from Boehringer-Mannheim Corporation (Indianapolis, IN). Tissue culture medium (CMRL-1066) and 1 M Hepes were from Gibco Co. (Grand Island, NY). Newborn bovine serum was from Hazleton Biologics (Lenexa, KS). β-TC3 cells (passage 34) were obtained through the University of Pennsylvania Diabetes Center from Dr. D. Hanahan (University of California, San Francisco). The following compounds were purchased from Sigma Chemical Co. (St. Louis, MO): D-glucose, carbachol, Hanks' balanced salt solution, penicillin, streptomycin, glutamine, Ficoll, fatty acid free bovine albumin, and 1-stearoyl-2-arachidonyl-*sn*-glycerol. Phospholipid standards were obtained from Serdary Research Laboratories (Port Huron, MI). Neutral lipids and fatty acids were purchased from NuChek Prep (Elysian, MN). Organic solvents (HPLC grade or Optima grade) were provided by Fisher Scientific Co. (Pittsburgh, PA). RHC-80267 [1,6-bis[[[(cyclohexylideneamino)oxy]carbonyl]amino]-hexane] was purchased from Biomol (Plymouth Meeting, PA). Other chemicals (except as indicated) were purchased from Sigma or Fisher. 1-Stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol (0.55 mCi/mmol), 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine, and [^{3}H]phosphatidylinositol were obtained from Amersham (Arlington Heights, IL). 1-Palmitoyl-2-[9,10-^{3}H]palmitoyl-*sn*-glycero-3-phosphocholine was obtained from New England Nuclear (Wilmington, DE). Silica gel G TLC 20 × 20 cm plates were obtained from Analtech (Newark, DE).

Methods

Isolation of Islets and β-TC3 Cell Line Culture. Islets were isolated from male Sprague−Dawley rats as previously described (Konrad et al., 1992a). Typically, this procedure provided 350−400 islets per rat, which were then used as described here. β-TC3 cells were cultured in T75 or T162 flasks in the presence of RPMI 1640 (11 mM glucose) supplemented with 10% fetal bovine serum, penicillin (75 μg/mL), streptomycin (50 μg/mL), and 2 mM L-glutamine. Cells were trypsinized and subcloned weekly, and medium was changed twice weekly and the day prior to fractionation. The insulin secretory capacity of the β-TC3 cells in response to glucose and carbachol was monitored regularly. Cells were used between passages 40 and 55.

Preparation of Islet Homogenates. Freshly isolated islets (1000−2000) were homogenized with 10−20 manual strokes in a Potter-Elvehjem homogenizer in 0.1−1 mL of a 50 mM (pH 7.40) TES solution containing 1 mM EGTA. Protein concentration of the homogenates was calculated using a bicinchoninic acid microassay with bovine serum albumin as the standard (Smith et al., 1985). Homogenates were stored at −20 °C prior to use or used fresh.

Preparation of 2-Arachidonyl-sn-glycerol and 2-[^{14}C]-Arachidonyl-sn-glycerol. Ten milligrams of 1-stearoyl-2-

arachidonyl-*sn*-glycerol was resuspended in 10 mL of chloroform and separated into aliquots in 10 silanized 5 mL borosilicate tubes. To each tube was added a trace amount (0.01 μCi) of 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol. Chloroform was evaporated, and 1-stearoyl-2-arachidonyl-*sn*-glycerol (1 mg/tube) was resuspended in 100 μL of 50 mM (pH 7.40) TES supplemented with 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$. To each tube was added 2500 units of *Rhizopus arrhizus* lipase. Digestion was carried out in a shaking water bath at 37 °C for 15 min. The reaction was stopped by the addition of 2 mL of ice-cold chloroform/methanol (1:2, v/v). Lipids were then extracted and run on silica gel G plates in a solvent system of petroleum ether/diethyl ether/acetic acid (140:60:2, v/v/v). The spot on each lane corresponding to the monoacylglycerol standard (R_f 0.20) was scraped from the plate. Monoacylglycerol was eluted from the silica with three 1 mL washes of chloroform/methanol (1:2). The amount of 2-arachidonyl-*sn*-glycerol synthesized was quantitated by ester assay using a standard curve with cholesteryl acetate (Kates, 1986). Freshly synthesized 2-arachidonyl-*sn*-glycerol (0.1 mg/mL in chloroform) was stored at −70 °C prior to use. The purity of the monoacylglycerol was determined on a silica gel G plate presprayed with 10% boric acid and developed in chloroform/acetone/methanol (96:4:2, v/v/v). Greater than 90% of the monoacylglycerol was 2-arachidonyl-*sn*-glycerol on the basis of its migration in this system (R_f 0.22). 2-[^{14}C]Arachidonyl-*sn*-glycerol was prepared by digesting 10 μCi of 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol with 2500 units of *Rhizopus arrhizus* lipase.

Diacylglycerol Lipase and Monoacylglycerol Lipase Assays. Incubations were carried out in silanized 13 × 100 round-bottom tubes containing 85 μL of 50 mM TES (pH 7.40) supplemented with either 1 mM EGTA or 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$ and 10 μL of enzyme source. The reaction was then started by the addition of 5 μL of the appropriate radioactive substrate dissolved in ethanol. The typical final concentrations of diacylglycerol and monoacylglycerol were 2 mM and 10 μM, respectively. Tubes were vortex mixed for 1 min on a multitube vortex mixer and sonicated in ice for 5 min. The tubes were incubated at 37 °C for 5 min in a shaking water bath. The reaction was stopped by the addition of 2 mL of ice-cold chloroform/methanol (1:2, v/v) supplemented with 0.25% of the antioxidant butylated hydroxytoluene. Samples were extracted and analyzed by TLC as described here.

Phospholipase A_2 Assay. Incubations were carried out as previously described (Jolly et al., 1993). The reaction was started by the addition of 5 μL of either 1-stearoyl-2-[^{14}C]-arachidonyl-*sn*-glycero-3-phosphocholine or 1-palmitoyl-2-[^{3}H]palmitoyl-*sn*-glycero-3-phosphocholine dissolved in ethanol (final concentration 2 μM). Tubes were vortex mixed for 1 min and incubated at 37 °C for 15 min in a shaking water bath.

Extraction of Fatty Acids and Phospholipids. Prior to extraction, carrier amounts (5 μg) of phospholipids, neutral lipids, and fatty acids were added to each tube to aid in recovery, followed by 1 mL of chloroform. Tubes were vortex mixed (1 min), sonicated (30 min, 4 °C), and vortex mixed (1 min). Tubes were centrifuged in a refrigerated table-top centrifuge (15 min, 4 °C, 800*g*). The lower phase was then transferred with a silanized Pasteur pipet to a clean, silanized 13 × 100 round-bottom borosilicate tube. The remaining aqueous upper phase was reextracted twice with chloroform (1 mL), and the extracts were combined with the previous organic phase. The organic phase was washed with water (1 mL), evaporated under nitrogen in a Turbovap (Zymark), and resuspended in 25 μL of chloroform.

One-Dimensional TLC Analysis of Monoacylglycerol and Fatty Acids. Samples were spotted onto the preadsorbent zone of channelled silica gel G TLC 20 × 20 cm plates (Analtech, Newark, DE) that had been activated for 30 min at 110 °C. Plates were developed for 30−45 min in petroleum ether/diethyl ether/acetic acid (140:60:2, v/v/v). The radioactivity of the chromatogram was quantitated with a Berthold linear analyzer 284 (Nashua, NH) equipped with a position-sensitive proportional high-resolution counter tube (200 mm long, 1380 V) continuously flushed (0.5 L/min) with P10 gas (90% argon/10% methane) and a 4 mm entrance window. Each TLC lane was scanned simultaneously in its entirety (20 cm) for 30 min. The instrument detected peaks as small as 50−100 dpm with a resolution of 0.5 mm. Data analysis was performed using version 7.19 of the 1D-TLC software. Radioactive peaks corresponding to free fatty acids and diacylglycerol were integrated by peak fitting (which automatically calculated and subtracted the background from adjacent regions). Peak identity was assigned by comparison with iodine-stained cold standards and radiolabeled commercial [^{3}H]arachidonic acid. Typically, the following R_f values were obtained: phospholipids, 0; monoacylglycerol, 0.19; diacylglycerol, 0.45; arachidonic acid, 0.63; triacylglycerol, 0.88. In order to calculate enzyme activity, the integrated counts in either monoacylglycerol or arachidonate were expressed as a percentage of the total radioactivity on the lane. This percentage was then converted to the amount of phospholipid, diacylglycerol, or monoacylglycerol hydrolyzed by multiplying it by the mass of substrate originally present in each reaction tube. Enzyme activity was then normalized to the amount of protein present and expressed as the apparent specific activity (mass of substrate hydrolyzed/milligram of protein).

Phospholipase C Assay. Incubations were carried out in silanized 13 × 100 round-bottom tubes containing 200 μL of 50 mM HEPES (pH 7.0) with 50 μg of phosphatidylinositol, 0.5 μCi of [^{3}H]phosphatidylinositol, 0.1 mM dithiothreitol, 1 mM EGTA, 3 mM $CaCl_2$, and 1% deoxycholate. Reactions were started by the addition of 10 μL of enzyme source. The tubes were incubated for 10 min at 37 °C in a shaking water bath. Reactions were stopped with the addition of 1 mL of chloroform/methanol/HCl (100:100:6, v/v/v) and 0.3 mL of 1 N HCl containing 5 mM EGTA. Tubes were vortex mixed and centrifuged, and the radioactivity in the aqueous phase was quantitated in a scintillation counter (Rhee et al., 1991).

Subcellular Fractionation of β-TC3 Cells. β-TC3 cells were fractionated by sequential centrifugation using a sucrose-based gradient as previously described (McDaniel et al., 1983), with minor modifications. Eight to ten confluent T162 flasks of β-TC3 cells were washed three times with 50 mM MES (pH 7.2), 250 mM sucrose, and 1 mM EGTA and resuspended in 1 mL of the same medium. Cells were then mechanically homogenized in a Potter-Elvehjem homogenizer, and the homogenate was centrifuged (600*g*, 10 min) on a Sorvall centrifuge. The supernatant was collected in a silanized Corex 16 × 100 tube and centrifuged again (20000*g*, 20 min, JA-20 rotor, Beckman L5-50B centrifuge). The resulting supernatant was centrifuged at

150000*g* (TL-100.3 rotor, Beckman TL-100 centrifuge) for 90 min to yield an endoplasmic reticulum-enriched pellet and cytosol. The pellet obtained on the 20000*g* centrifugation was resuspended in 500 μL of 10 mM MES (pH 7.4), layered over a discontinuous sucrose gradient (1 mL each of densities 1.14, 1.16, 1.18, and 1.20), and centrifuged at 150000*g* (SW-40 rotor, Beckman L5-50 ultracentrifuge) for 90 min. Band 1 in this scheme corresponds to plasma membrane. Bands 3 and 4 correspond to a mixed fraction of mitochondria and secretory granules. Enzyme marker studies with 5′-nucleotidase show that the plasma membrane fraction is enriched 23-fold compared to the homogenate.[2]

Incubation of Intact Islets for Insulin Secretion. Islets (25 per condition) were placed in siliconized 10 × 75 tubes. Islets were preincubated in 1 mL of Krebs−Hepes buffer (25 mM Hepes (pH 7.40), 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% bovine serum albumin) supplemented with 3 mM glucose for 30 min at 37 °C in a shaking water bath under an atmosphere of 95% O_2/5% CO_2. The medium was removed from each tube and replaced with the appropriate secretagogue. Incubation was continued for 30 min, at which time a sample of the supernatant was removed for insulin measurement by radioimmunoassay (Konrad et al., 1992a).

Fatty Acid Analysis by Gas Chromatography−Electron-Capture Detection. Islets (150−200 per condition) were placed in silanized 13 × 100 round-bottom borosilicate tubes with Teflon screw-caps. Islets were preincubated in 1 mL of Krebs−Hepes buffer supplemented with 3 mM glucose and fatty acid free bovine serum albumin (0.1%) for 30 min at 37 °C in a shaking water bath under an atmosphere of 95% O_2/5% CO_2. The medium was then removed from each tube and replaced with the appropriate secretagogue. Incubation was then continued for 30 min. A sample of the supernatant was removed for insulin RIA, and the reaction was stopped by the addition of 2 mL of ice-cold chloroform/methanol (1:2, v/v) supplemented with 0.25% of the antioxidant butylated hydroxytoluene. The tubes were then chilled for 15 min in a dry ice/ethanol bath and stored at −20 °C.

Prior to extraction, 3 nmol of an internal standard (heptadecanoic acid, C17:0) was added to each tube, followed by 1 mL of chloroform. Tubes were vortex mixed (1 min), sonicated (30 min, 4 °C), and vortex mixed (1 min). Samples were then extracted and run on TLC as described earlier. The location of the fatty acid spot was identified by running radioactive standards on adjacent lanes, which were localized by radioactivity scanning. The silica was scraped and fatty acids were eluted with three washes (1 mL) of chloroform/methanol (1:2, v/v). Chloroform/methanol was then evaporated in a Savant concentrator. To dried fatty acids were added 30 μL of acetonitrile, 10 μL of 35% pentafluorobenzyl bromide in acetonitrile, and 10 μL of diisopropylethylamine. Tubes were incubated with gentle shaking at room temperature for 10 min. Samples were then evaporated in a Savant concentrator and reconstituted in 1 mL of heptane.

Sample analysis was performed on a capillary gas chromatography system consisting of a Varian 3400 gas chromatograph with a Varian 1093 septum-equipped programmable (SPI) injector, a Varian 1077 split/splitless capillary injector, a flame ionization detector, and a ^{63}Ni electron-capture detector (Varian Chromatography, Walnut Creek, CA). Data analysis was performed on an IBM-compatible computer (486DX2 chip, 50 MHz) running the Varian GC Star Workstation software (version A.2). Derivatized fatty acids were analyzed on a 25 m SGE BPX70 column (Fisher) with 0.25 μm film thickness and 0.33 nm internal diameter, at a head pressure of 7 psi using nitrogen as the carrier gas. On-column injection was performed with the SPI injector equipped with a silanized high-performance insert (Restek Corporation, Bellefonte, PA) and a low-bleed Thermolite septum. After injection, the injector was programmed from 65 to 165 °C at 150 °C/min and from 165 to 300 °C at 50 °C/min. The electron-capture detector was maintained at 300 °C. The oven temperature was held at 85 °C for 2 min, increased to 165 °C at 40 °C/min, then to 250 °C at 3.5 °C/min, and held for 1 min at 250 °C. Peak identity was assigned by the retention times, which were obtained by comparison to authentic fatty acid pentafluorobenzyl esters, which were determined systematically before each set of analyses. The fatty acid pentafluorobenzyl esters were quantitated relative to heptadecanoic acid pentafluorobenzyl ester. Any signal derived from blank samples was routinely subtracted.

Glucose Oxidation. Groups of 100 islets were placed in silanized 13 × 100 round-bottom borosilicate tubes and preincubated in 1 mL of Krebs−Hepes buffer supplemented with 3 mM glucose for 30 min at 37 °C in a shaking water bath under an atmosphere of 95% O_2/5% CO_2. The medium was removed from each tube and replaced with 0.5 mL of Krebs−Hepes buffer supplemented with 3 mM [^{14}C]glucose or 17 mM [^{14}C]glucose/0.5 mM carbachol ± 35 μM RHC-80267. Tubes were gassed with 95% O_2/5% CO_2, capped with rubber stoppers with a polypropylene inner cup containing a piece of filter paper, and incubated for an additional 60 min at 37 °C. The reaction was stopped by injecting 0.3 mL of methylbenzethonium in methanol (Sigma) onto the filter paper and 0.5 mL of 1 N HCl onto the islets. The tubes were incubated at room temperature in a shaking water bath for 2 h to trap $^{14}CO_2$ on the filter paper. Trapped $^{14}CO_2$ was quantitated by counting in a liquid scintillation spectrometer (Weaver et al., 1978; Konrad et al., 1992b).

Other Methods. Free calcium concentrations were maintained by the addition of EGTA and were calculated as previously described (Wolf et al., 1986). Free calcium concentrations were systematically checked with an Orion calcium electrode, which was calibrated between 10^{-7} and 10^{-3} M as previously described (Wolf et al., 1986). Insulin radioimmunoassay was performed by the University of Pennsylvania Diabetes Endocrine Research Center. RHC-80267 was dissolved in a final concentration of 0.1% DMSO.

Data Analysis. Results are expressed as the mean ± SE. Statistical analysis was performed using version 5.0 of SSPS for Windows (SSPS Inc., Chicago, IL). Data were analyzed by one-way or two-way analysis of variance followed by multiple comparisons between means using the least significant difference test or by analysis of covariance where appropriate. A probability of $p < 0.05$ was considered statistically significant. K_m and V_{max} of diacylglycerol lipase and monoacyl glycerol lipase were estimated by double-reciprocal plot and linear regression analysis using the curve-fitting program FigP (Biosoft, St. Louis, MO).

[2] R. J. Konrad, C. Major, Y. C. Jolly, and B. A. Wolf, manuscript in preparation.

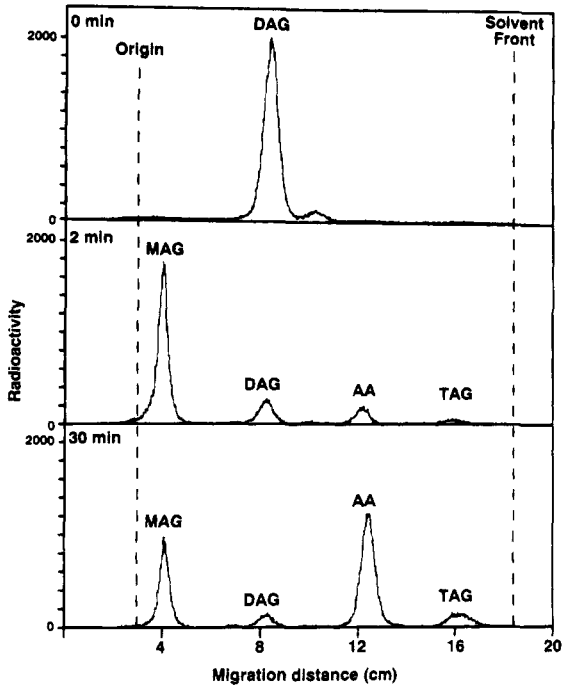

FIGURE 1: Linear analyzer quantitation of islet diacylglycerol hydrolysis. Islet homogenates were incubated in silanized tubes for various times with 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol in 50 mM TES, 1 mM EGTA, and 2 μM 1-stearoyl-2-arachidonyl-*sn*-glycerol. The reaction was stopped by the addition of 2 mL of ice-cold chloroform/methanol (1:2, v/v) supplemented with 0.25% butylated hydroxytoluene. Lipids were extracted as described in the Experimental Procedures section and analyzed by one-dimensional TLC with a mobile phase of petroleum ether/diethyl ether/acetic acid (140:60:2, v/v/v). Radioactivity on the plates was quantitated with a Berthold linear analyzer and is expressed as the counts of radioactivity as a function of migration distance on the plate. MAG, monoacylglycerol; DAG, diacylglycerol; AA, arachidonic acid; TAG, triacylglycerol.

RESULTS

Diacylglycerol and Monoacylglycerol Hydrolysis in Islets. Diacylglycerol hydrolysis was first measured by incubating islet homogenates with 2 μM 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol, followed by extraction and analysis of labeled products by TLC. As shown by the chromatograms in Figure 1, at early time points, the first product to appear was 2-[^{14}C]-arachidonyl-*sn*-glycerol. Only at later time points did a significant amount of free [^{14}C]arachidonate accumulate. This suggested that islet diacylglycerol hydrolysis occurs in a two-step process. The first step is the hydrolysis of stearate in the *sn*-1 position by diacylglycerol lipase, giving rise to 2-arachidonyl-*sn*-glycerol. The second and rate-limiting step is the hydrolysis of arachidonate at the *sn*-2 position of monoacylglycerol by monoacylglycerol lipase, giving rise to unesterified arachidonate.

These enzyme activities were characterized further. As shown in Figure 2, diacylglycerol lipase, the enzyme that hydrolyzes stearate from the *sn*-1 position of diacylglycerol, had an apparent K_m of 0.86 $\pm$ 0.12 mM and a V_{max} of 3.2 $\pm$ 0.1 μmol/mg/min. In addition, this enzyme showed a 51% increase in activity when the calcium concentration was increased from 0.1 to 100 μM. In the presence of 2.5 mM calcium, enzyme activity was greater than in the absence of calcium from pH 6 to 9. Characteristics of monoacylglycerol lipase are shown in Figure 3. This enzyme, which hydrolyzes arachidonate from the *sn*-2 position of monoacylglycerol, was calcium-independent. Enzyme activity increased as the pH was increased from pH 5 to 9. Monoacylglycerol lipase had a lower apparent K_m of 0.14 $\pm$ 0.03 μM and a V_{max} of 4.8 $\pm$ 0.3 nmol/mg/min.

In comparison to islets, rat exocrine pancreas had 3.7 times more diacylglycerol lipase activity (4.38 versus 1.19 of μmol diacylglycerol hydrolyzed/mg of protein/min, $n = 4$). After collagenase digestion, islets were purified on a discontinuous Ficoll gradient, cleaned extensively, and hand-picked. We estimate that there is less than 1% exocrine contamination of the islet preparation. Thus, it is unlikely that any exocrine tissue remaining associated with the islets significantly contributed to islet diacylglycerol lipase activity.

Subcellular Distribution of Diacylglycerol and Monoacylglycerol Lipases. To assess the cellular distribution of enzyme activity, subcellular fractions were prepared from β-TC3 cells. β-TC3 cells represent a previously characterized β-cell line that secretes insulin robustly in response to glucose and carbachol (Baffy et al., 1993). Use of β-TC3 cells has the advantage that large quantities of tissue are available for fractionation, whereas islets yield a limited amount of tissue. Incubation of homogenates made from subcellular fractions with 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol and 2-[^{14}C]arachidonyl-*sn*-glycerol demonstrated that the plasma membrane was the fraction most enriched in both diacylglycerol lipase and monoacylglycerol lipase activity (Figure 4).

Effect of RHC-80267 on Insulin Secretion and Diacylglycerol Lipase Activity. The data presented thus far indicated that a two-step pathway for the hydrolysis of diacylglycerol clearly existed in islets. Furthermore, the two enzymes that act sequentially to hydrolyze diacylglycerol were found to be most enriched in the plasma membrane of insulin-secreting β-TC3 cells. These data suggested a possible role for diacylglycerol hydrolysis in insulin secretion. In order to assess the functional importance of diacylglycerol hydrolysis for insulin secretion, the diacylglycerol lipase inhibitor RHC-80267 was employed. Figure 5A shows the effect of RHC-80267 on glucose- and carbachol-stimulated insulin secretion from intact islets. RHC-80267 clearly inhibited 28 mM glucose- and 0.5 mM carbachol-stimulated insulin secretion in a dose-dependent manner, while not affecting baseline 3 mM glucose insulin secretion. The inhibition of secretion was half-maximal at a concentration of 10 μM and virtually complete at 35 μM ($p < 0.05$ versus control). RHC-80267 at a concentration of 35 μM also virtually abolished the increase in insulin secretion observed when islets were stimulated with 28 mM glucose alone (Figure 6).

As shown in Figure 5B–D, RHC-80267 specifically inhibited diacylglycerol lipase activity in islet homogenates in a dose-dependent manner. Inhibition was half-maximal at a concentration of 10 μM and virtually complete at 35 μM ($p < 0.05$ versus control). Monoacylglycerol lipase and phospholipase C activities were not significantly affected by RHC-80267. Phospholipase A_2 activity, as measured by the production of either [^{14}C]arachidonate from 1-stearoyl-2-[^{14}C]-arachidonyl-*sn*-glycero-3-phosphocholine or [^{3}H]palmitate from 1-palmitoyl-2-[^{3}H]palmitoyl-*sn*-glycero-3-phosphocholine, was not significantly inhibited by 35 μM RHC-80267 [198 $\pm$ 19 pmol of substrate hydrolyzed/mg of protein for control versus 182 $\pm$ 24 pmol/mg with 35 μM RHC-80267 present using the substrate 1-palmitoyl-2-[^{3}H]palmitoyl-*sn*-

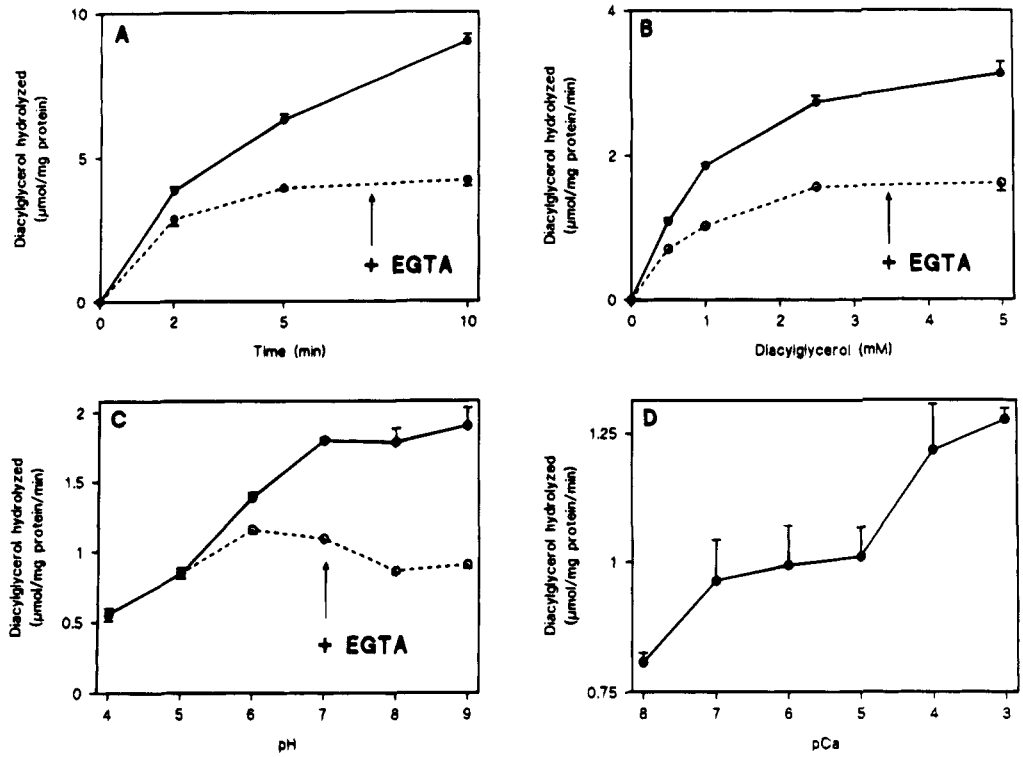

FIGURE 2: Characterization of islet diacylglycerol lipase. Islet homogenates were incubated with 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol in 50 mM TES containing 5% ethanol and either 1 mM EGTA or 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$. The concentration of 1-stearoyl-2-arachidonyl-*sn*-glycerol used was 2 mM, except where noted. Panel A: Time course of diacylglycerol hydrolysis. Panel B: Dose curve of diacylglycerol hydrolysis. The concentration of 1-stearoyl-2-arachidonyl-*sn*-glycerol used was 0.1−5 mM. Panel C: pH dependency of diacylglycerol hydrolysis. At pH 6 and below, MES was substituted for TES, and at pH 8 and above, Tris was substituted for TES. Panel D: Calcium dependency of diacylglycerol hydrolysis. The calcium concentration in the presence of 1 mM EGTA was varied and measured using a calcium-sensitive electrode. Results in panels A−C are shown as the mean ± SE from duplicate observations per point, representative of two experiments. Results in the final panel are shown as the mean ± SE from triplicate observations representative of three experiments.

glycero-3-phosphocholine, $n = 3$; 359 ± 41 pmol/mg for control versus 316 ± 20 pmol/mg with 35 μM RHC-80267 present using the substrate 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine, $n = 3$]. Importantly, glucose oxidation in islets was not significantly affected by RHC-80267, as demonstrated in Table 1. This indicated that the effect of RHC-80267 to inhibit glucose-induced increases in fatty acids and insulin secretion was not merely due to inhibition of glucose oxidation.

Effect of RHC-80267 on Secretagogue-Induced Arachidonic Acid Accumulation and Insulin Secretion. In this set of experiments, insulin secretion was again markedly inhibited by 35 μM RHC-80267, as shown in Figure 7A. Figure 7B shows the effect of RHC-80267 on arachidonic acid accumulation. In the absence of 35 μM RHC-80267, stimulation of islets with 28 mM glucose and 0.5 mM carbachol caused an 8.0-fold increase in unesterified arachidonic acid (from 0.37 ± 0.16 to 2.99 ± 0.53 pmol/islet, $p < 0.05$ versus control). When islets were stimulated with 28 mM glucose and 0.5 mM carbachol with RHC-80267 present, the normal 8-fold increase in arachidonic acid was reduced to 3.1-fold (1.02 ± 0.22 pmol/islet, $p < 0.05$ versus control). Inhibition was also observed when islets were stimulated with 28 mM glucose alone or 0.5 mM carbachol alone. Glucose (28 mM) alone caused an increase in arachidonate from 0.44 ± 0.07 to 2.14 ± 0.41 pmol/islet that was reduced to 0.32 ± 0.10 pmol/islet in the presence of 35 μM RHC-80267 ($n = 2$). Carbachol (0.5 mM) alone caused an increase in arachidonate from 0.44 ± 0.07 to 1.68 ± 0.61 pmol/islet that was reduced to 0.48 ± 0.08 pmol/islet in the presence of 35 μM RHC-80267 ($n = 2$).

Table 2 shows the effect of 28 mM glucose and 0.5 mM carbachol on the levels of fatty acids other than arachidonate. The predominant unesterified fatty acids detected were stearate, palmitate, arachidonate, and oleate. Minor amounts of myristate and linoleate were also quantitated. In the absence of RHC-80267, glucose and carbachol significantly increased, by 5−6-fold, the levels of these fatty acids (Table 2). In the presence of 35 μM RHC-80267, however, secretagogue-induced increases in the various classes of unesterified fatty acids were significantly reduced to less than a 2-fold increase. In particular, the secretagogue-induced increase in unesterified palmitate and oleate was completely abolished by the diacylglycerol lipase inhibitor RHC-80267, which is consistent with our previous reports that glucose-stimulated diacylglycerol is enriched in these fatty acids (Wolf et al., 1989, 1990).

DISCUSSION

We have shown that a two-step pathway exists in islets for the hydrolysis of diacylglycerol and the subsequent production of unesterified arachidonic acid. In the first step, the *sn*-1 bond of 1-stearoyl-2-arachidonyl-*sn*-glycerol was hydrolyzed by a diacylglycerol lipase, producing 2-arachidonyl-*sn*-glycerol. Next, the *sn*-2 bond of 2-arachidonyl-*sn*-glycerol was hydrolyzed by a monoacylglycerol lipase to yield arachidonic acid. Both of these enzymes were highly enriched in the plasma membrane of insulin-secreting β-cells,

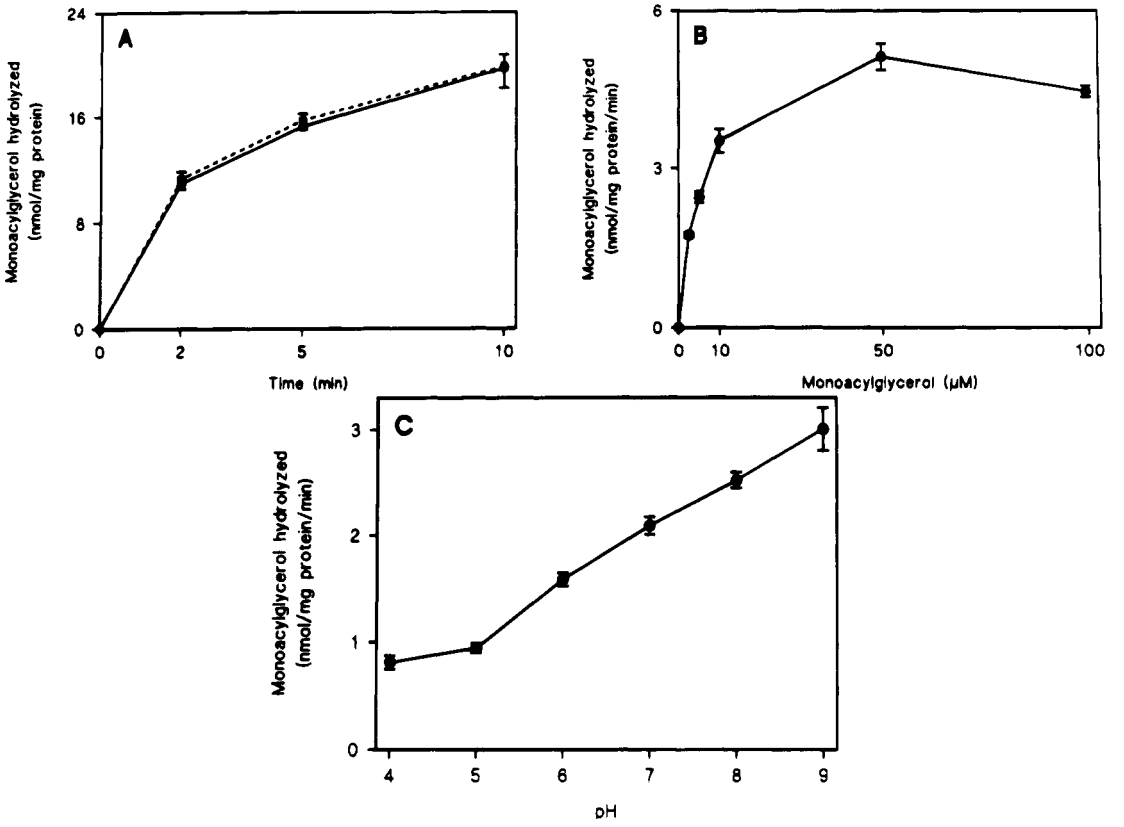

FIGURE 3: Characterization of islet monoacylglycerol lipase. Islet homogenates were incubated with 2-[^{14}C]arachidonyl-*sn*-glycerol in 50 mM TES containing 5% ethanol and either 1 mM EGTA or 2.5 mM $CaCl_2$ and 1 mM $MgCl_2$. The concentration of 2-arachidonyl-*sn*-glycerol used was 10 μM, except where noted. Panel A: Time course of monoacylglycerol hydrolysis. Panel B: Dose curve of monoacylglycerol hydrolysis. The concentrations of 2-arachidonyl-*sn*-glycerol used were 0, 2.5, 5, 10, 50, and 100 μM. Panel C: pH dependency of monoacylglycerol hydrolysis. At pH 6 and below, MES was substituted for TES, and at pH 8 and above, Tris was substituted for TES. Results are shown as the mean ± SE from triplicate observations per point.

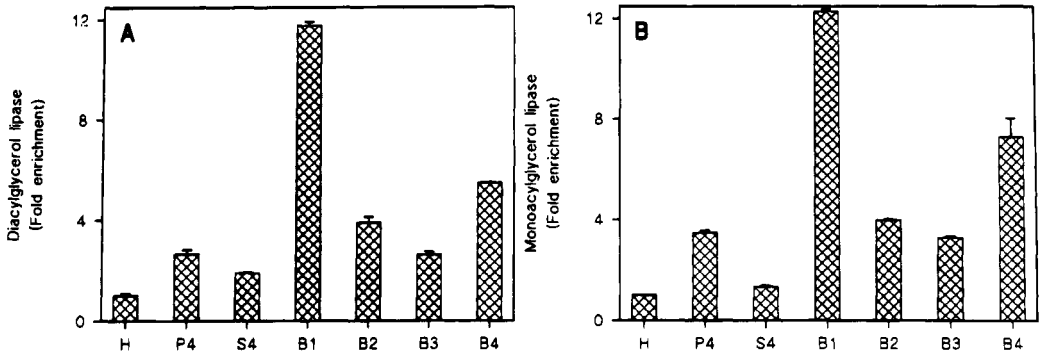

FIGURE 4: Subcellular distributions of diacylglycerol lipase and monoacylglycerol lipase in insulin-secreting β-TC3 cells. Insulin-secreting β-TC3 cells were fractionated as described in the Experimental Procedures, and fraction homogenates were incubated with the appropriate substrates as described in Figures 2 and 3. The specific activity of diacylglycerol and monoacylglycerol lipases in the homogenate and in each of the subcellular fractions was determined and the fold enrichment was calculated. Panel A: Subcellular distribution of diacylglycerol lipase. Panel B: Subcellular distribution of monoacylglycerol lipase. Results are shown as the mean ± SE from duplicate observations representative of two experiments. H, homogenate; P4, endoplasmic reticulum; S4, cytosol; B1, plasma membrane; B2, intermediate fraction; B3, mitochondria; B4, secretory granules.

which is the site of diacylglycerol production by phospholipase C. Importantly, we have also demonstrated that selective inhibition of islet diacylglycerol lipase by RHC-80267 markedly inhibited both glucose- and carbachol-induced increases in arachidonic acid, as well as glucose- and carbachol-induced insulin release.

Diacylglycerol hydrolysis in islets was quantitated to determine whether diacylglycerol is an important source of unesterified fatty acids, such as arachidonic acid. The production of arachidonic acid from diacylglycerol previously has been identified in islet homogenates using 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol as a substrate (Schrey & Montague, 1983). In this study, the free [^{14}C]arachidonate produced was used as an index of diacylglycerol hydrolysis, but monoacylglycerol production was not quantitated. Therefore, the assumption was made that arachidonate is hydrolyzed directly from the *sn*-2 position of diacylglycerol. In contrast, our observations clearly demonstrate that, in islets, 1-stearoyl-2-arachidonyl-*sn*-glycerol is first converted to 2-arachidonyl-*sn*-glycerol. Hydrolysis of arachidonate from 2-arachidonyl-*sn*-glycerol then occurs and constitutes the rate-limiting step in diacylglycerol hydrolysis.

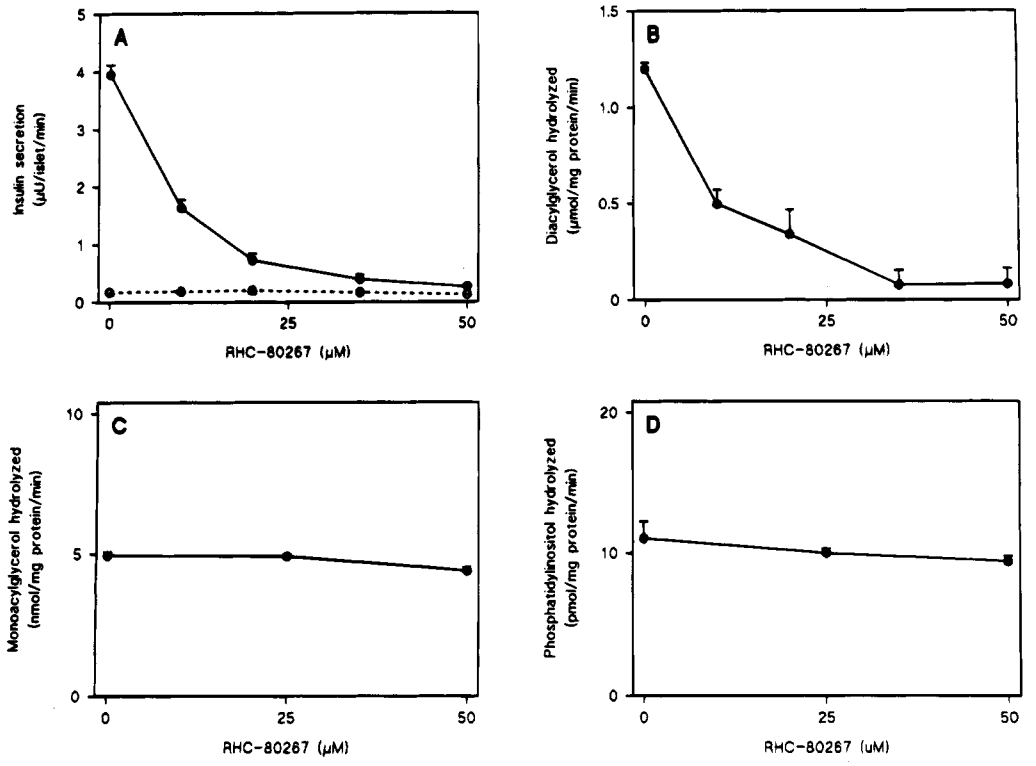

FIGURE 5: Effect of the diacylglycerol lipase inhibitor RHC-80267 on insulin secretion, islet diacylglycerol lipase, monoacylglycerol lipase, and phospholipase C. Panel A: Effect of RG-80267 on insulin secretion. Islets (25 per condition) were stimulated with either 3 mM glucose (○) or 28 mM glucose and 0.5 mM carbachol (●) ± varying concentrations of RHC-80267 for 30 min. Results are expressed in microunits/islet/minute. Panel B: Effect of RHC-80267 on islet diacylglycerol lipase. Islet homogenates were incubated with 1-stearoyl-2-[^{14}C]arachidonyl-*sn*-glycerol, as described in Figure 2, with 0−50 μM RHC-80267. Panel C: Effect of RHC-80267 on islet monoacylglycerol lipase. Islet homogenates were incubated with 2-[^{14}C]arachidonyl-*sn*-glycerol, as described in Figure 2, with 0−50 μM RHC-80267. Panel D: Effect of RHC-80267 on islet phospholipase C. Islet homogenates were incubated with [^{3}H]phosphatidylinositol, as described in the Experimental Procedures, with 0−50 μM RHC-80267. Results in panel B are shown as the mean ± SE from quadruplicate observations representative of two experiments. Results in all other panels are shown as the mean ± SE from quadruplicate observations.

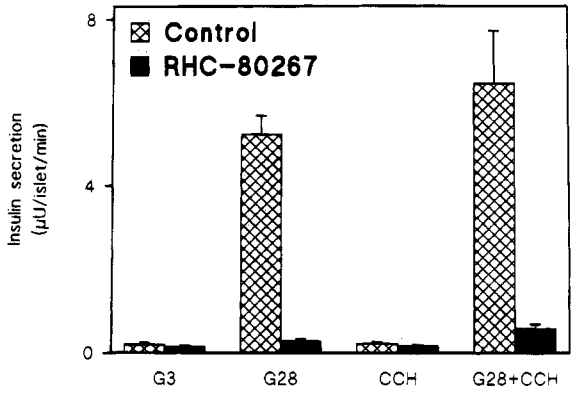

FIGURE 6: Effect of the diacylglycerol lipase inhibitor RHC-80267 on secretagogue-induced insulin secretion by isolated islets. Insulin secretion induced by 3 mM glucose (G3), 28 mM glucose (G28), 0.5 mM carbachol (CCH), and 28 mM glucose and 0.5 mM carbachol (G28/CCH) ± 35 μM RHC-80267 was measured as described for Figure 5.

Table 1: Effect of RHC-80267 on Islet Glucose Oxidation[a]

conditions	glucose oxidation (pmol/islet/h)
3 mM glucose	7.85 ± 0.76
17 mM glucose + 0.5 mM carbachol	32.38 ± 4.53[b]
3 mM glucose + 35 μM RHC-80267	7.58 ± 0.80
17 mM glucose + 0.5 mM carbachol + 35 μM RHC-80267	31.40 ± 3.31[b]

[a] Groups of 100 islets were incubated for 60 min with either 3 mM [^{14}C]glucose or 17 mM [^{14}C]glucose and 0.5 mM carbachol, as described in the Experimental Procedures. Results are shown as the mean ± SE from eight observations from two experiments. [b] $p < 0.05$ versus 3 mM glucose control.

Relatively little is known about diacylglycerol hydrolysis in other cell types. Neural-enriched cultures from fetal mouse spinal cord have been shown to possess both diacylglycerol lipase and monoacylglycerol lipase activity. Both enzymes have been shown to be stimulated by glutamate, as well as by bradykinin, in a time-dependent manner, suggesting an important role for these enzymes (Farooqui et al., 1990, 1993). Human platelets have also been shown to possess both diacylglycerol lipase and monoacylglycerol lipase activity. In platelet microsomes, diacylglycerol lipase was found to be the rate-limiting enzyme (Chau & Tai, 1988). Similarly, in human neutrophils, diacylglycerol hydrolysis to yield arachidonic acid was also found to occur in a two-step process, with diacylglycerol lipase again being the rate-limiting enzyme. In human neutrophils, both enzymes were localized to gelatinase-containing granules and translocated to the plasma membrane when the neutrophils were stimulated by the calcium ionophore A23187 (Balsinde et al., 1991). An important difference in islets compared to neutrophils and platelets is that the monoacylglycerol lipase appears to catalyze the rate-limiting step of diacylglycerol hydrolysis.

Several earlier studies in islets have shown that insulin secretion induced by the fuel secretagogue D-glucose and the muscarinic secretagogue carbachol is accompanied by the accumulation of islet unesterified arachidonic acid.

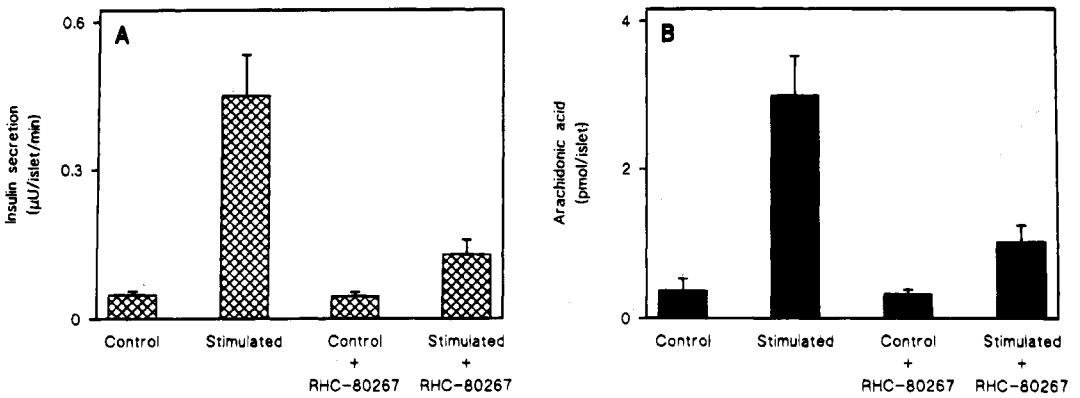

FIGURE 7: Effect of the diacylglycerol lipase inhibitor RHC-80267 on insulin secretion and arachidonic acid accumulation in isolated islets, as measured by gas chromatography–electron capture detection. Panel A: Effect of RHC-80267 on insulin secretion. Islets (150–200 per condition) were preincubated in 1 mL of Krebs–Hepes buffer supplemented with 3 mM glucose for 30 min at 37 °C under an atmosphere of 95% O_2/5% CO_2. The medium was removed from each tube and replaced with either 3 mM glucose or 28 mM glucose and 0.5 mM carbachol ± 35 μM RHC-80267. The incubation was continued for an additional 30 min and insulin release was measured. All incubation solutions contained 0.1% DMSO. Panel B: Effect of RHC-80267 on arachidonic acid accumulation. Each tube received 2 mL of ice-cold chloroform/methanol (1:2, v/v) supplemented with 0.25% of the antioxidant butylated hydroxytoluene and 3 nmol of heptadecanoic acid as an internal standard. Unesterified arachidonic acid was extracted, purified by TLC, derivatized to its pentafluorobenzyl ester, and analyzed by gas chromatography–electron capture detection as described in the Experimental Procedures. Results are shown as the mean ± SE of insulin secretion (microunits/islet/minute) and arachidonic acid accumulation (picomoles/islet) from six observations per condition from two experiments.

Table 2: Effect of the Diacylglycerol Lipase Inhibitor RHC-80267 on Fatty Acid Accumulation in Intact Islets[a]

conditions	fatty acid (pmol/islet)			
	C16:0	C18:0	C18:1	total
3 mM glucose	0.73 ± 0.20	1.84 ± 0.45	0.35 ± 0.28	3.42 ± 0.66
28 mM glucose + 0.5 mM carbachol	4.18 ± 1.16[b]	6.52 ± 0.30[b]	1.66 ± 0.51[b]	17.23 ± 3.41[b]
3 mM glucose + 35 μM RHC-80267	2.13 ± 1.27	1.59 ± 0.51	0.89 ± 0.48	5.22 ± 2.44
28 mM glucose + 0.5 mM carbachol + 35 μM RHC-80267	2.93 ± 0.57	3.57 ± 0.54[b,c]	1.24 ± 0.38	10.17 ± 1.17[b,c]

[a] Islets (150–200 per condition) were preincubated and then incubated as described for Figure 7. Results are shown as the mean ± SE of fatty acid accumulation from six observations per condition from two experiments. Total fatty acid represents the sum of palmitate (C16:0), stearate (C18:0), oleate (C18:1), arachidonate (from Figure 7), and other minor unesterified fatty acids. [b] $p < 0.05$ versus 3 mM glucose control. [c] $p < 0.05$ versus 28 mM glucose + 0.5 mM carbachol control.

Arachidonic acid was measured by gas chromatography–mass spectrometry, by the release of [^{3}H]arachidonic acid, or by immunoassay of PGE_2 production as an index of arachidonate accumulation (Wolf et al., 1986, 1987, 1991; Jolly et al., 1993; Ramanadham et al., 1993; Konrad et al., 1992a, 1993).

Arachidonic acid is an attractive candidate for part of a signal transduction mechanism for insulin secretion from the β-cell (Turk et al., 1993). Arachidonic acid mobilizes Ca^{2+} from the endoplasmic reticulum of the β-cell and stimulates insulin release under certain conditions (Wolf et al., 1986, 1987). In addition, arachidonic acid, at the concentrations present in islets, facilitates voltage-dependent Ca^{2+} entry in the β-cell (Wolf et al., 1991; Turk et al., 1993). Also, arachidonic acid recently has been shown to stimulate protein kinase C in other cell types (Shinomura et al., 1991; Nishizuka, 1992). Importantly, we have previously shown that arachidonic acid accumulation in islets stimulated with glucose and carbachol occurs as rapidly as insulin release (Konrad et al., 1992a). Increases in both arachidonate and insulin secretion clearly are present after a 2 min lag (Konrad et al., 1992a). In light of these observations, we attempted to assess the contribution, if any, of diacylglycerol hydrolysis to arachidonate accumulation during insulin secretion.

There are two major pathways by which insulin secretagogues can generate arachidonic acid. One pathway involves phospholipase A_2 hydrolysis of membrane phospholipids. In islets, there are at least three phospholipase A_2 activities: a calcium-dependent membrane-bound activity stimulated by millimolar amounts of Ca^{2+}, a calcium-dependent cytosolic enzyme stimulated by micromolar amounts of Ca^{2+}, and a calcium-independent plasmalogen-selective phospholipase A_2 (Jolly et al., 1993; Gross et al., 1993). There is a combination of biochemical and pharmacological data suggesting that these phospholipases A_2 are involved in insulin secretion (Metz, 1991; Turk et al., 1993). In intact islets, the muscarinic agonist carbachol (but not glucose) stimulates the accumulation of arachidonic acid and lysophosphatidylcholine (the end products of phospholipase A_2 activation) (Konrad et al., 1992a). More recently, we have shown that carbachol and glucose transiently activate calcium-dependent membrane and cytosolic phospholipases A_2 (Jolly et al., 1993). A calcium-independent plasmalogen-selective phospholipase A_2 activity has also been shown to exist in islets (Gross et al., 1993). Specific inhibition of this enzyme inhibits glucose-induced insulin secretion (Ramanadham et al., 1993). The relative contribution of each type of phospholipase A_2 to arachidonic acid production is unsettled at the present time.

We now show that another source of agonist-induced accumulation of arachidonic acid is from diacylglycerol, a product of phospholipase C activation in islets. The diacylglycerol lipase inhibitor RHC-80267 has previously been shown to inhibit glucose-induced insulin secretion (Capito

et al., 1989). Interpretation of these previous studies, however, has been obscured by questions concerning the possibility that RHC-80267 may interfere with glucose metabolism (Wolf et al., 1990; Capito et al., 1989). Glucose-induced insulin secretion clearly requires glucose oxidation, which results in membrane depolarization and an influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels (Matschinsky, 1990; Meglasson & Matschinsky, 1986; Malaisse et al., 1979; Wollheim & Sharp, 1981). Obviously, because of the importance of glucose oxidation in insulin secretion, compounds that inhibit glucose oxidation cannot be used to selectively probe a potential signal transduction pathway. In our hands, RHC-80267 did not interfere with glucose oxidation, as measured by the production of $^{14}CO_2$ from [^{14}C]glucose. RHC-80267 proved to be quite specific for diacylglycerol lipase and did not significantly inhibit islet phospholipase C, monoacylglycerol lipase, or phospholipase A_2. Our data strongly suggest that diacylglycerol hydrolysis is essential for agonist-induced insulin secretion since the dose dependencies of RHC-80267 inhibition of insulin secretion and diacylglycerol lipase are similar (Figure 5,B).

Both glucose and carbachol stimulate phospholipase C in islets (Henquin & Nenquin, 1988; Verspohl et al., 1990; Wolf et al., 1988b, 1989; Peter-Riesch et al., 1988; Turk et al., 1986; Biden et al., 1987). Stimulation of phospholipase C generates the second messengers Ins(1,4,5)P_3, which mobilizes intracellular Ca^{2+}, and 1,2-*sn*-diacylglycerol, which stimulates protein kinase C. It is widely believed that the generation of these second messengers is important in glucose-induced insulin release and is required for muscarinic-induced insulin secretion. The role of diacylglycerol and protein kinase C in insulin secretion is controversial. We and others have failed to detect a glucose-induced increase in diacylglycerol mass in islets, although there was a change in the fatty acyl composition of diacylglycerol (Wolf et al., 1989, 1990; Peter-Riesch et al., 1988). Carbachol, however, clearly stimulates diacylglycerol accumulation (Regazzi et al., 1990; Turk et al., 1993). In addition, although carbachol stimulation of protein kinase C and phosphorylation of its endogenous MARCKS substrate have been demonstrated by several groups, glucose stimulation of protein kinase C is not readily observed (Wollheim & Regazzi, 1990; Turk et al., 1993). Note, however, that one group has convincingly shown that glucose causes the translocation of the α-isoform of protein kinase C and stimulates MARCKS phosphorylation in freshly isolated islets (Ganesan et al., 1990; Calle et al., 1992). One possibility that could explain this apparent discrepancy is that diacylglycerol produced by glucose stimulation of phospholipase C is rapidly metabolized to unesterified fatty acids. In previous studies, we have quantitated the total diacylglycerol content of islets by mass spectrometric and enzymatic methods as approximately 1 pmol/islet, which corresponds to a minimal concentration of 250 μM assuming equal intracellular distribution. Since both diacylglycerol lipase and monoacylglycerol lipase are greatly enriched in the plasma membrane of insulin-secreting β-cells (Figure 4), and since the apparent K_m of diacylglycerol lipase (0.86 mM) is 3 orders of magnitude greater than that of monoacylglycerol lipase, it is likely that any diacylglycerol formed would be rapidly hydrolyzed.

Studies with the halo enol lactone suicide substrate inhibitor of plasmalogen-selective phospholipase A_2 have shown complete suppression of secretagogue-induced eicosanoid and insulin release (Ramanadham et al., 1993). There are several potential explanations for this apparent discrepancy. In those studies, arachidonic acid release was measured indirectly by quantitating the accumulation of its cyclooxygenase metabolite, PGE_2. Islet PGE_2 levels after glucose and carbachol stimulation were 1 and 3 fmol/islet (Ramanadham et al., 1993), which are 1000 times less than the islet arachidonic acid levels reported in this and previous studies (Wolf et al., 1986, 1991). It is conceivable that glucose activation of the calcium-independent plasmalogen-selective phospholipase A_2 contributes to a quantitatively small but physiologically important pool of arachidonic acid.

The relative contribution of the phospholipase C/diacylglycerol lipase/monoacylglycerol lipase pathway to secretagogue-induced arachidonic acid accumulation in islets can be grossly estimated from our measurements of islet arachidonic acid levels by gas chromatography with electron-capture detection, a method that has femtomole sensitivity (Major & Wolf, 1994). For the combination of glucose and carbachol, this pathway accounts for approximately two-thirds of arachidonic acid accumulation and secretagogue-induced insulin secretion (Figure 7). Our data demonstrate that the hydrolysis of diacylglycerol is an important source of glucose- and carbachol-induced accumulation of arachidonic acid and is essential for glucose- and muscarinic-stimulated insulin secretion.

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