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Free Fatty Acid Accumulation in Secretagogue-Stimulated Pancreatic Islets and Effects of Arachidonate on Depolarization-Induced Insulin Secretion†

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ABSTRACT: Free fatty acids in isolated pancreatic islets have been quantified by gas chromatography–mass spectrometry after stimulation with insulin secretagogues. The fuel secretagogue D-glucose has been found to induce little change in islet palmitate levels but does induce the accumulation of sufficient unesterified arachidonate by mass to achieve an increment in cellular levels of 38–75 μM. Little of this free arachidonate is released into the perifusion medium, and most remains associated with the islets. Glucose-induced hydrolysis of arachidonate from islet cell phospholipids is reflected by release of the arachidonate metabolite prostaglandin E_2 (PGE_2) from perifused islets. Both the depolarizing insulin secretagogue tolbutamide (which is thought to act by inducing closure of β-cell ATP-sensitive K^+ channels and the influx of extracellular Ca^{2+} through voltage-dependent channels) and the calcium ionophore A23187 have also been found to induce free arachidonate accumulation within and PGE_2 release from islets. Surprisingly, a major fraction of glucose-induced eicosanoid release was found not to require Ca^{2+} influx and occurred even in Ca^{2+}-free medium, in the presence of the Ca^{2+}-chelating agent EGTA, and in the presence of the Ca^{2+} channel blockers verapamil and nifedipine. Exogenous arachidonic acid was found to amplify the insulin secretory response of perifused islets to submaximally depolarizing concentrations of KCl, and the maximally effective concentration of arachidonate was 30–40 μM. These observations suggest that glucose-induced phospholipid hydrolysis and free arachidonate accumulation in pancreatic islets are not simply epiphenomena associated with Ca^{2+} influx and that arachidonate accumulation may play a role in the signaling process which leads to insulin secretion.

The β cells of pancreatic islets can be induced to secrete insulin by a variety of secretagogues. D-Glucose is an example of a fuel secretagogue and must be metabolized in order to induce secretion (Malaisse et al., 1979; Hedeskov, 1980; Ashcroft, 1980; Wollheim & Scharp, 1981; Meglasson & Matschinsky, 1986). Tolbutamide and other hypoglycemic sulfonylureas are depolarizing secretagogues which appear to interact primarily with an ATP-sensitive K^+ channel in the β-cell plasma membrane and to induce closure of this channel, which results in membrane depolarization (Henquin & Meissner, 1982; Sturgess et al., 1985; Trube et al., 1986).

Stimulation of islets from rodents and from humans with glucose has been found to result in the rapid generation of mediators derived from the hydrolysis of membrane phospholipids, including arachidonic acid and its metabolites and inositol trisphosphates (Prentki & Matschinsky, 1987; Biden et al., 1987; Turk et al., 1987a; Robertson, 1988; Metz, 1988b). Such substances participate in the transduction of extracellular signals into cellular responses in other systems (Berridge, 1987; Needleman et al., 1986) and might be suspected to play a similar role in β cells (Turk et al., 1987a; Metz, 1988b). In particular, D-glucose has been found to induce the accumulation of substantial amounts of unesterified, unmetabolized arachidonic acid in islets with a time course that closely parallels that of glucose-induced insulin secretion (Wolf et al., 1987a,b; Turk et al., 1987b). At the concentrations which accumulate in glucose-stimulated islets (Turk et al., 1987b), arachidonate has also been found to induce the release of Ca^{2+} sequestered in islet endoplasmic reticulum (Wolf et al., 1987a,b). This effect is exerted by arachidonic acid itself and not by one of its metabolites (Wolf et al., 1987a). It is not yet known whether glucose induces accumulation of free fatty acids other than arachidonate in islets. Other unsaturated, but not saturated, fatty acids have been found to mimic the Ca^{2+}-mobilizing effect of arachidonate in other tissues (Beaumier et al., 1987; Knepel et al., 1988; Naccache et al., 1989; Tohmatsu et al., 1989; Chan & Turk, 1987). These fatty acids might, if present in sufficient concentrations, also participate in the regulation of the islet cytosolic Ca^{2+} levels and the secretion of insulin.

The islet endoplasmic reticulum is believed to play an important role in maintaining the cytosolic Ca^{2+} concentration in the range of 100 nM (Prentki et al., 1984), and stimulation of islets with insulin secretagogues, including glucose, has been

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demonstrated to induce a rise in cytosolic [Ca^{2+}] (Gylfe, 1988a,b). Recent evidence indicates, however, that the dominant event in producing the fuel secretagogue-induced rise in islet cytosolic [Ca^{2+}] is Ca^{2+} entry from the extracellular space rather than Ca^{2+} release from internal sequestration sites (Arkhammar et al., 1987). In addition, glucose-induced hydrolysis of membrane phosphoinositides appears to be triggered by Ca^{2+} influx (Biden et al., 1987), which could be taken to suggest that hydrolysis of islet membrane phospholipids is a secondary event with no primary role in the induction of insulin secretion.

This Ca^{2+} entry appears to occur via voltage-sensitive plasma membrane Ca^{2+} channels, which become active when the β cell depolarizes from its negative resting potential (Keahey et al., 1989). The resting membrane potential in the islet β cells, as in many other excitable cells, is a K^+ potential (Hille, 1984). The magnitude of this potential is directly proportional to the plasma membrane permeability to potassium and is inversely proportional to the extracellular potassium concentration. The β-cell plasma membrane K^+ permeability is determined by ATP-sensitive K^+ channels, and stimulation of islets with fuel secretagogues, such as glucose, induces closure of these channels (Cook & Hales, 1984). Secretagogue-induced closure of the β-cell K^+ channel causes the membrane potential to rise toward zero. As the membrane potential rises, voltage-sensitive plasma membrane Ca^{2+} channels are activated and permit entry of Ca^{2+} from the extracellular space. Recently, arachidonic acid has been reported to facilitate activation of voltage-dependent Ca^{2+} channels in a GH_3 pituitary tumor cell line (Vacher et al., 1989). Arachidonate concentrations in the range of 10–40 μM were found to shift the voltage dependence for Ca^{2+} channel opening to lower voltages in the GH_3 cells. These observations suggest the hypothesis that arachidonate might act to amplify the rise of cytosolic [Ca^{2+}] produced by submaximal depolarization of islet β cells and thereby to amplify insulin secretion.

The studies described here examine the effect of the fuel secretagogue D-glucose on the accumulation of arachidonate and other fatty acids in isolated pancreatic islets, the effects of nonfuel secretagogues which promote calcium entry (tolbumatide and A23187) on islet eicosanoid release, the role of extracellular calcium entry on D-glucose-induced eicosanoid release, and the influence of arachidonate on depolarization-induced insulin secretion from islets.

Experimental Procedures

Male Sprague-Dawley rats (180–220-g body weight) were purchased from Sasco (O'Fallon, MO). Collagenase was obtained from 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). D-Glucose was purchased from the National Bureau of Standards (Washington, DC). Ionophore A23187, tolbutamide, verapamil, and nifedipine were obtained from Sigma Chemical Co. (St. Louis, MO).

Isolation and Culture of Islets. Islets were isolated aseptically from male Sprague-Dawley rats fed ad libitum, as described elsewhere (McDaniel et al., 1983). 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 D-glucose). This procedure typically yielded 300 islets per rat. Isolated islets were then cultured overnight in four petri dishes with 2.5 mL of complete CMRL-1066 medium at 24 °C under an atmosphere of 95% air–5% CO_2.

Incubation of Islets. Isolated, cultured islets were washed 3 times in Hepes–Krebs–3 mM glucose (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). For static incubations, the islets were hand-counted under stereomicroscopic visualization into silanized 13 mm × 100 mm borosilicate tubes. From 50 to 600 islets were employed per condition, depending on the abundance of the analytes to be measured in a given experiment. The islets were then preincubated for 30 min with shaking in 1.0 mL of Hepes–Krebs–3 mM glucose at 37 °C under an atmosphere of 95% air–5% CO_2. The preincubation medium was then removed, discarded, and replaced with 1.0 mL of incubation medium containing D-glucose (3 or 17 mM) and, where appropriate, an additional agent (e.g., 4 μM A23187 or 200 μM tolbutamide). The incubation medium had been prewarmed to 37 °C. The incubation was then continued for the appropriate period (2–30 min) with shaking at 37 °C under an atmosphere of 95% air–5% CO_2. Incubations were terminated by removing the medium (for measurement of secreted insulin, PGE_2, and free fatty acids) and adding 2 mL of ice-cold methanol to the islet pellet, from which free fatty acids were extracted and analyzed as described below.

Perfusion of Islets. Isolated islets were hand-counted onto the Millipore filter of each chamber of a quadruple chamber perifusion apparatus and were perifused with Hepes–Krebs–3 mM glucose for 30 min at 37 °C at a rate of 1 mL/min, as described elsewhere (Lacy et al., 1972). The composition of the medium was then changed to contain a higher glucose concentration (17 mM) or to contain another agonist or an inhibitor (e.g., 5–30 μM verapamil or 0.2–2 μM nifedipine), and perifusion was then continued for 10–30 min. Perifusion effluent was collected continuously in aliquots of 1–5 mL and was subsequently analyzed for content of insulin, PGE_2, and free fatty acids, as described below. At the end of the perifusion period, the filter containing the islets was removed from the perifusion chamber and plunged into a 16 mm × 100 mm silanized borosilicate tube containing 2 mL of ice-cold methanol. The tube was then immersed in a solid CO_2–propanol bath, and the islet pellets were subsequently processed and analyzed for free fatty acid content as described below.

Insulin Measurement by Radioimmunoassay. One hundred microliters of the aqueous incubation medium from each experimental condition was placed into a 12 mm × 75 mm borosilicate tube and stored at −20 °C until the time of radioimmunoassay. Appropriate dilutions were then prepared, and insulin was measured by double-antibody radioimmunoassay employing ^{125}I-labeled insulin as described elsewhere (Wright et al., 1971).

Prostaglandin E_2 Measurement by Enzyme Immunoassay. Prostaglandin E_2 was extracted from incubation medium essentially as described (Powell, 1982). In brief, 400 μL of the aqueous incubation medium from each condition was diluted

with 100 μL of ethanol. The pH was adjusted to 3.0 with 1.0 N HCl. This solution was applied to a disposable octadecylsilicic acid column (1 mL; Baker Scientific, Phillipsburg, NJ) that had previously been conditioned by elution with 3 mL of ethanol and then 3 mL of H_2O. The columns were then washed with successive 3-mL aliquots of ethanol–H_2O (1:9), petroleum ether, and petroleum ether–$CHCl_3$ (65:35) by application of negative pressure. PGE_2 was then eluted from the column with methyl formate (3 mL) and analyzed by enzyme immunoassay (EIA) as described (Pradelles et al., 1985). In brief, the methyl formate solution containing the PGE_2 was concentrated to dryness under N_2 and reconstituted in buffer containing 100 mM potassium phosphate, 1.5 mM sodium azide, 400 mM NaCl, 1 mM Na_4-EDTA, and 0.1% BSA, pH 7.4. Aliquots (50 μL) of each sample were then placed in wells of a microtiter plate (VWR Scientific, Batavia, IL) that had been previously coated with mouse monoclonal anti-rabbit IgG (2 mg/mL; Biomol Laboratories, Philadelphia, PA). A tracer consisting of PGE_2 labeled with acetylcholinesterase (50 μL; Cayman Chemical, Ann Arbor, MI) and rabbit anti-PGE_2 antibody (50 μL, Biomol) were then added, and the plate was incubated at room temperature overnight. The plate was then washed (0.005% Tween in 100 mM potassium phosphate buffer, pH 7.4), and 200 μL of a developing reagent [700 mM acetylthiocholine iodide and 500 mM 5,5'-dithiobis(2-nitrobenzoic acid) in 100 mM potassium phosphate buffer] was added to each well. The plate was then covered with cellophane and agitated for 120 min. The absorbance at 414 nm was then determined on a Flow Systems microtiter plate reader, and the mass of PGE_2 in each well was calculated by interpolation from a standard curve prepared on the same plate. Fitting of the standard curve and calculation of the quantity of PGE_2 in the samples were performed with software provided by AIA reagents (Denver, CO) through the courtesy of Dr. Robert Murphy and Dr. Jacques Maclouf.

Fatty Acid Extraction, Purification, and Derivatization. To each 2 mL of aqueous supernatant was added 4 mL of methanol containing 100 ng of heneicosanoic acid (C21:0 fatty acid from NuChek Prep, Elysian, MN) as an internal standard. To each islet pellet in 2 mL of methanol was added an additional 2 mL of methanol containing 100 ng of heneicosanoic acid and 2 mL of H_2O. All samples were then vortexed (1 min), sonicated at room temperature (30 min), vortexed again (1 min), and centrifuged (3000 rpm × 15 min). The supernatants were transferred with a silanized Pasteur pipet to a silanized 10-mL, conical, screw-cap borosilicate tube and were acidified to approximately pH 3.5 with 1 N HCl (0.25 mL). CH_2Cl_2 (2 mL) containing 0.25 mg/mL butylated hydroxytoluene (BHT) as an antioxidant was then added, and the tubes were vortexed (1 min) and centrifuged (3000 rpm, 5 min). The CH_2Cl_2 (lower) phase was then transferred to a 5-mL conical, screw-cap, silanized borosilicate tube. The residual aqueous phase was extracted with a second 2-mL aliquot of CH_2Cl_2 containing BHT. That extract was combined with the first, and water (2 mL) was added. The tubes were then vortexed (1 min) and centrifuged (5 min, 3000 rpm). The aqueous (upper) phase was discarded, and the residual organic (lower) phase was washed twice more with H_2O (2 mL). The extracts were then concentrated to dryness under N_2, transferred to an acid-washed, silanized, conical, screw-cap, 1-mL ReactiVial (Pierce, Rockford, IL) with ethanol, concentrated to dryness again, the reconstituted in ethanol (100 μL). The samples were then applied to prescored, channeled silica gel G thin-layer chromatographic (TLC) plates (250 μm; Analtech, Newark, DE) with preabsorbent lanes. The TLC plates were then developed in solvent system A (petroleum ether–diethyl ether–acetic acid, 70:30:1, v/v). Free fatty acids of chain length C14 to C22 migrated close together in this system with an R_f of approximately 0.34 and were clearly distinguished from *sn*-1,2-diacylglycerol (R_f 0.21), triacylglycerol (R_f 0.65), and phosphatidylcholine (origin). Standard erucic acid (C22:1) applied to outer lanes of the plates was used to locate the fatty acid spot and was visualized with iodine vapor. Silicic acid containing the fatty acids was scraped from each lane of the plate individually into silanized, 10-mL, conical, screw-cap vials, and the fatty acids were eluted from the silicic acid by vortexing (5 min) in methanol (1 mL). The silicic acid was removed by centrifugation (3000 rpm, 15 min). The supernatant was transferred to an acid-washed, silanized, conical, 1-mL ReactiVial and concentrated to dryness. The residue was reconstituted in methanol (0.1 mL). The fatty acids were then coverted to their methyl ester derivatives by treatment with ethereal diazomethane (room temperature, 5 min). Samples were then concentrated to dryness under N_2 and reconstituted in heptane (20 μL) before GC analysis.

Gas Chromatographic and Mass Spectrometric Analysis of Fatty Acid Methyl Esters. Samples were introduced into a Hewlett-Packard 5890 gas chromatograph via a Grob-type injector operated in the splitless mode and were analyzed on an HP-1 capillary column (cross-linked methylsilicone, 12-m length, 0.2-mm i.d., 0.33-μm film thickness) with helium as the carrier gas (total flow 10 mL/min). The initial oven temperature was 85°C. At 0.5 min after injection, the splitless valve was actuated, and the oven temperature was increased at a rate of 30 °C/min to a temperature of 175 °C. The temperature was then increased at a rate of 10 °C/min to a temperature of 250 °C. Thereafter, the temperature was increased at a rate of 30 °C/min to a final temperature of 300 °C. The temperature of the injector was maintained at 250 °C and that of the transfer line at 280 °C. Under these conditions, the approximate retention times of the fatty acid methyl esters were as follows: myristate, 5.42 min; palmitate, 6.90 min; linoleate, 8.22 min; oleate, 8.30 min; stearate, 8.53 min; arachidonate, 9.53 min; and heneicosanoate, 10.98 min. In some cases, a flame ionization detection (FID) was employed, and the signal was integrated with a Hewlett-Packard 3393 A integrator. In most cases, detection was performed with a mass spectrometer (Hewlett-Packard 5970 mass selective detector) operated with a Hewlett-Packard RTE-A data system. Ions monitored for individual fatty acid methyl esters included m/z 242 (myristate), m/z 270 (palmitate), m/z 294 (linoleate), m/z 296 (oleate), m/z 296 (stearate), m/z 175 (arachidonate), and m/z 340 (heneicosanoate, the internal standard).

Results

To examine the free fatty acid content of islets, islet pellets were extracted with CH_2Cl_2–methanol in the presence of heneicosanoate as an internal standard. The free fatty acids were then separated from other lipids by thin-layer chromatography and converted to their methyl ester derivatives with diazomethane. The fatty acid methyl esters were then analyzed by capillary column gas chromatography–mass spectrometry with selected ion monitoring. The fatty acids were identified by their GC retention times and by their characteristic molecular ion or by a strong fragment ion. Each of the individual endogenous, free fatty acids from the islets was quantified relative to the internal standard heneicosanoate, which was added in a known amount to the original sample.

Islets were found to contain measurable and similar quantities of the free fatty acids palmitate (C16:0), linoleate

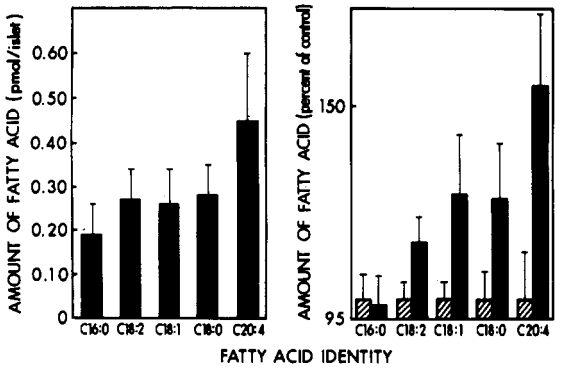

FIGURE 1: Free fatty acids in isolated pancreatic islets: relative abundance and effects of stimulation with glucose. Seven hundred islets per condition were placed in silanized 13 mm × 100 mm borosilicate test tubes and preincubated for 30 min at 37 °C in the presence of 3 mM glucose, as described under Experimental Procedures. The medium was then removed and replaced with fresh medium containing either 3 or 17 mM glucose, and the islets were incubated for an additional 30 min at 37 °C. The left panel indicates the absolute amounts of the individual fatty acids in the islets. The right panel indicates the change in the relative abundance of the fatty acids induced by stimulation with 17 mM glucose. In the right panel, data with 3 mM glucose are depicted by hatched bars, and data for 17 mM glucose are depicted by solid bars. Standard errors of the mean are indicated (n = 6).

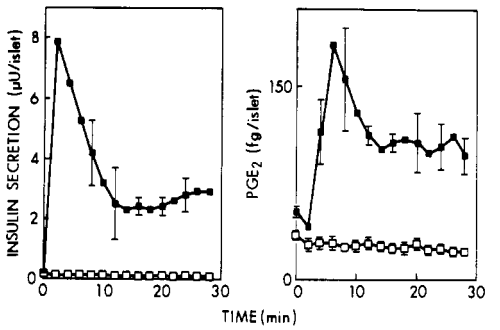

FIGURE 2: Glucose-induced eicosanoid release and insulin secretion by perifused islets. Five hundred islets each were placed in individual perifusion chambers and were perifused at 1 mL/min for 30 min at 37 °C with 3 mM glucose. Perifusion effluent during this period was discarded. The islets were then perifused for an additional 30 min either with 3 mM glucose (open symbols) or with 17 mM glucose (closed symbols), and the perifusion effluent was collected in 2-mL aliquots during this period for the measurement of insulin secretion by radioimmunoassay (left panel) and for PGE_2 measurement by enzyme immunoassay (right panel) as described under Experimental Procedures. Standard errors of the mean are indicated (n = 6). Statistical analysis by two-way ANOVA indicated a highly significant effect of 17 mM glucose compared to 3 mM glucose on both insulin secretion (F = 43.02, $p < 0.0001$) at all time points after 2 min and on PGE_2 release (F = 161.02, $p < 0.0001$) at all time points after 4 min.

(C18:2), oleate (C18:1), and stearate (C18:0) and slightly more free arachidonate (C20:4), as illustrated in Figure 1 (left panel). It is of interest that the free (unesterified) amounts of the polyunsaturated fatty acids arachidonate and linoleate were 2.6% and 3.4%, respectively, of the known islet glycerolipid content of these fatty acids (Turk et al., 1986a,b), while the free amounts of the saturated or monounsaturated fatty acids palmitate, stearate, and oleate were only 0.6%, 1.0%, and 1.2%, respectively, of the islet glycerolipid content of these fatty acids (Turk et al., 1986a,b).

Exposure of islets in static (30 min) incubations to a maximally stimulatory concentration of D-glucose (17 mM) induced a significant (p = 0.03) rise in the islet content of free arachidonate, as illustrated in Figure 1 (right panel), which is consistent with an earlier report (Wolf et al., 1987a). Under these conditions, there was no change in free palmitate levels in the islets and a smaller rise in the levels of the 18-carbon fatty acids, which did not achieve statistical significance. The rise in mass of free arachidonate in glucose-stimulated islets was sufficient to effect a change in the cellular concentration of arachidonate of 38–75 μM, assuming an islet intracellular volume of 2–4 nL (Turk et al., 1987a).

Perifusion of islets on filters in chambers through which there is continuous flow of medium permits assessment of the dynamics of release of materials which escape from the islet into the extracellular space. In addition, insulin secretion and some biochemical responses to secretagogue stimulation are sometimes more robust with perifused islets than with static incubations, particularly when large numbers of islets are used. This phenomenon is thought to reflect accumulation of inhibitory mediators, such as somatostatin (Kanatsuka et al., 1984), in the medium of static incubations, while such mediators are continuously removed in perifusions. Perifused islets stimulated with 17 mM D-glucose were found not to release detectable amounts of free fatty acids into the perifusion medium, although a rise in the islet content of free arachidonate to a level (2.13 ± 0.26)-fold higher than that of control islets incubated with 3 mM glucose was observed when the islets from the filter of the perifusion chambers were extracted and analyzed for free fatty acid content at the end of a 10-min perifusion period. Little change in the islet free palmitate content occurred under these conditions [the value from 17 mM D-glucose-stimulated islets was (1.28 ± 0.26)-fold that of control islets incubated with 3 mM D-glucose], although there was a robust (9-fold) insulin secretory response to glucose (not shown).

The dynamics of insulin secretion from 17 mM D-glucose-stimulated, perifused islets are illustrated in Figure 2 (left panel) and include a rapidly rising and then falling first phase of secretion which is followed by a stable or slowly rising second phase of secretion. Although detectable amounts of free arachidonate are not released from the islets into the perifusion medium under these conditions, the generation of free arachidonate from hydrolysis of membrane phospholipids induced by 17 mM D-glucose is reflected by the release of the oxygenated arachidonate metabolite prostaglandin E_2 (PGE_2) into the perifusion medium, as illustrated in Figure 2 (right panel). As shown in the figure, PGE_2 release from islets perifused with a basal, nonstimulatory D-glucose concentration of 3 mM is quite stable with time. Stimulation of the islets with 17 mM D-glucose results in a rapid, biphasic release of PGE_2. Under these conditions, the enantiomer L-glucose at a concentration of 17 mM stimulated neither insulin secretion nor PGE_2 release above basal levels (not shown).

Both accumulation of arachidonate within islets and release of PGE_2 into the medium were also induced by the sulfonylurea secretagogue tolbutamide with a time course similar to the induction of insulin secretion, as illustrated in Figure 3. Tolbutamide is thought to induce secretion by reducing the β-cell plasma membrane permeability to K^+ (Sturgess et al., 1985), which results in membrane depolarization and the influx of extracellular Ca^{2+} through voltage-dependent channels. Many phospholipases are Ca^{2+}-activated or Ca^{2+}-dependent (Waite, 1985), and the hydrolysis of membrane phospholipid and accumulation of free fatty acids might be a consequence of Ca^{2+} influx. Ca^{2+} influx is also induced by the ionophore A23187, and this compound was also found to induce accumulation of free arachidonate within islets and release of PGE_2 into the medium (Figure 4).

Since glucose induces a rise in β-cell cytosolic [Ca^{2+}] predominantly by inducing Ca^{2+} influx and exerts little influence

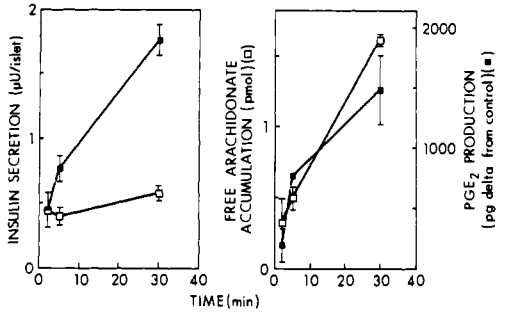

FIGURE 3: Tolbutamide-induced accumulation of arachidonate, release of PGE_2, and insulin secretion by isolated pancreatic islets. Five hundred islets each were placed into individual 13 mm × 100 mm silanized borosilicate tubes and incubated with 3 mM glucose for 30 min at 37 °C. The medium was then removed, discarded, and replaced with fresh medium containing 3 mM glucose and tolbutamide (200 μM). The islets were then incubated for an additional 2, 5, or 30 min at 37 °C. At the end of this period, the medium was then removed for determination of insulin secretion and PGE_2 release, as described in Figure 2, and for measurement of free fatty acid content, as described under Experimental Procedures. To the islet pellet was added 2 mL of ice-cold methanol containing 100 ng of heneicosanoic acid, and free fatty acids were extracted from the pellet and measured by GC–MS, as described under Experimental Procedures. The left panel indicates the measurments of secreted insulin. Open symbols in the left panel depict data obtained with 3 mM glucose, and closed symbols depict data obtained with tolbutamide (100 μM) plus 3 mM glucose. The right panel indicates the measurements of PGE_2 released into the medium (closed symbols) and of free arachidonic acid contained in the islet pellet (open symbols). Points indicate the mean values from two experiments, and bars indicate the range of observed values.

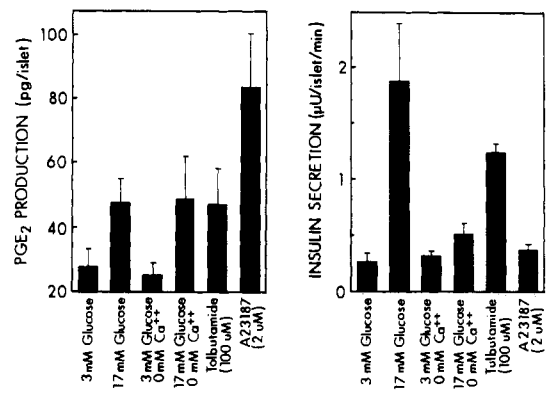

FIGURE 4: Extracellular calcium influx and eicosanoid release from isolated pancreatic islets. Fifty islets each were placed in individual 16 mm × 100 mm silanized borosilicate tubes and incubated with 3 mM glucose for 30 min or with 3 mM glucose and 2.5 mM Ca^{2+} at 37 °C. The medium was then removed and replaced with fresh medium containing (a) 3 mM glucose and 2.5 mM Ca^{2+} (b) 17 mM glucose and 2.5 mM Ca^{2+}, (c) 3 mM glucose and no Ca^{2+}, (d) 17 mM glucose and no Ca^{2+}, (e) tolbutamide (200 μM) plus 3 mM glucose and 2.5 mM Ca^{2+}, or (f) A23187 (2 μM) plus 3 mM glucose and 2.5 mM Ca^{2+}. The islets were then incubated for 10 min at 37 °C. At the end of this period, the medium was removed, and its contents of insulin and of PGE_2 were measured by radioimmunoassay and by enzyme immunoassay, respectively. Standard errors of the mean are indicated (n = 12). The p value for the increment in insulin secretion (vs 3 mM glucose plus 2.5 mM $CaCl_2$) induced by 17 mM glucose plus 2.5 mM $CaCl_2$ was 0.006, and that for tolbutamide was <0.001. Insulin secretion with 3 mM glucose was not significantly different between medium containing 2.5 mM $CaCl_2$ and calcium-depleted medium (p = 0.623), and 17 mM glucose failed to induce a significant rise in insulin secretion in calcium-depleted medium (p = 0.10 vs 3 mM glucose plus 2.5 mM $CaCl_2$). PGE_2 release induced by 17 mM glucose plus 2.5 mM $CaCl_2$ (p = 0.05 vs 3 mM glucose plus 2.5 mM $CaCl_2$) was not significantly different from that induced by 17 mM glucose in calcium-depleted medium (p = 0.951).

on the β-cell cytosolic [Ca^{2+}] in the absence of extracellular Ca^{2+} or in the presence of Ca^{2+} channel blockers (Rorsman et al., 1984), it was expected that glucose-induced arachidonate release would be prevented under the latter conditions. Removal of extracellular Ca^{2+} virtually completely inhibited glucose-induced insulin secretion in static incubations, as illustrated in Figure 4, right panel. Surprisingly, glucose-induced eicosanoid release was little affected by removal of Ca^{2+} from the extracellular medium and was similar in magnitude to that induced by glucose in Ca^{2+}-replete medium (Figure 4, left panel).

Similar experiments were performed in the presence of the calcium-chelating agent EGTA (1 mM) to completely remove extracellular Ca^{2+}. Under these conditions, 17 mM glucose still induced release of an amount of PGE_2 (47 ± 7 pg, n = 5) from islets that was not significantly different from that obtained in control, Ca^{2+}-replete medium in the absence of EGTA (54 ± 9 pg, n = 5). Basal PGE_2 production at 3 mM glucose was also essentially identical in Ca^{2+}-replete medium (22.4 ± 5.1 pg, n = 5) and in EGTA-containing medium (22.1 ± 2.0 pg, n = 5). In addition, the calcium channel blockers verapamil (5–30 μM) and nifedipine (0.2–2 μM) also failed to suppress glucose-induced PGE_2 release: The 17 mM glucose-stimulated PGE_2 production in the presence of 5 μM verapamil was (1.04 ± 0.13)-fold that of the drug-free control value, (1.11 ± 0.22)-fold the drug-free control value with 30 μM verapamil, (0.92 ± 0.18)-fold the drug-free control value with 200 nM nifedipine, and (1.24 ± 0.03)-fold the drug-free control value with 2 μM nifedipine. At these concentrations, verapamil and nifedipine suppress both glucose-induced islet phosphoinositide hydrolysis (Biden et al., 1987) and insulin secretion (Morgan et al., 1985).

These observations indicate that a substantial component of glucose-induced arachidonate release occurs by mechanisms that are independent of Ca^{2+} influx from the extracellular space. Arachidonate itself has recently been reported to facilitate activation of voltage-dependent Ca^{2+} channels in GH_3 pituitary tumor cells (Vacher et al., 1989). This suggests the possibility that arachidonate might act to amplify the β-cell rise in cytosolic [Ca^{2+}] produced by submaximal depolarization and thereby to amplify insulin secretion. This hypothesis was tested by examining the effect of arachidonate on insulin secretion induced by depolarizing concentrations of KCl in the extracellular medium.

Islets incubated with 4 mM glucose exhibit a basal insulin secretory rate of about 0.3 microunits $islet^{-1}$ min^{-1} when incubated with a physiologic concentration of extracellular K^+ (5 mM), as illustrated in Figure 5. Because the β-cell resting membrane potential is essentially a potassium potential, increasing the extracellular K^+ concentration results in membrane depolarization. This in turn induces entry of Ca^{2+} from the extracellular space through voltage-sensitive calcium channels. As illustrated in Figure 5, this is reflected by an increasing rate of insulin secretion at K^+ concentrations of 20 and 40 mM (open squares), although little increment in secretion occurs at 10 mM K^+. In the presence of 20 μM arachidonic acid (closed squares), a significantly augmented insulin secretory rate is observed at 10 mM (p = 0.022) and at 20 mM KCl (p = 0.005), although neither the basal insulin secretory rate nor the highest stimulated insulin secretory rate at 40 mM KCl is significantly affected by arachidonate. The arachidonate concentration dependence of this effect to amplify K^+-induced secretion at a submaximally stimulatory concentration (20 mM) of K^+ is illustrated in Figure 6. A significant (p < 0.05) effect is observed at 10 μM arachidonate and a maximal effect at 30 μM arachidonate. The concentration response curve appears to plateau or to assume a bell shape in the range 30–40 μM. It is striking that the data described in Figure 1 above indicate that increments in the free ara-

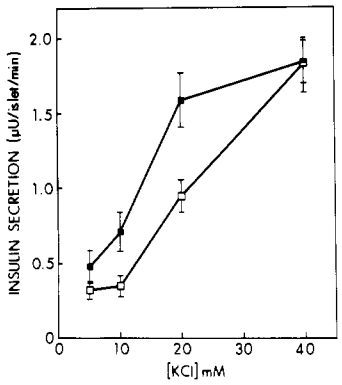

FIGURE 5: Influence of arachidonic acid on K^+-induced insulin secretion from isolated pancreatic islets. Isolated islets that had been cultured overnight were washed 3 times in KRB containing 4 mM glucose and 0.1% bovine serum albumin, and then 30 islets per condition were hand-counted under stereomicroscopic visualization into each of 45 (13 mm × 100 mm) silanized borosilicate tubes. The islets were washed 3 times with and then suspended in 0.2 mL of albumin-free KRB containing 4 mM glucose and were then preincubated for 30 min at 37 °C with shaking under an atmosphere of 95% air–5% CO_2. At the end of this period, the medium was removed and replaced with 0.2 mL of fresh medium that had been prewarmed to 37 °C and which contained 4 mM glucose and the desired concentration of KCl (5, 10, 20, or 40 mM) without (open symbols, controls) or with (closed symbols) arachidonic acid (20 μM). The islets were then incubated for 25 min at 37 °C with shaking under an atmosphere of 95% air–5% CO_2. At the end of this period, incubations were terminated by the addition of 0.2 mL of ice-cold KRB containing 0.2% bovine serum albumin, and the tubes were placed on ice. The medium was then removed for measurement of insulin by radioimmunoassay. Standard errors of the mean are indicated (n = 15). The p values for the difference between insulin secretion in the presence or absence of arachidonate were 0.202 at 5 mM KCl, 0.022 at 10 mM KCl, 0.005 at 20 mM KCl, and 0.945 at 40 mM KCl.

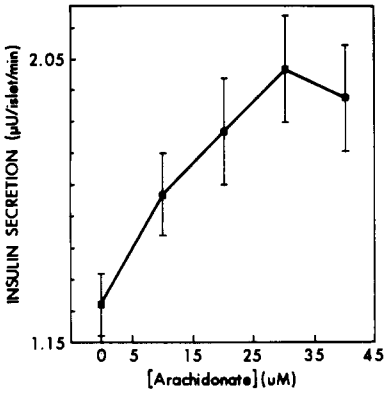

FIGURE 6: Concentration dependence of the amplification of K^+-induced insulin secretion by arachidonic acid. Experiments were performed as described in Figure 5, except that after completion of the preincubation period, the medium added for the experimental incubation contained 4 mM glucose, a fixed concentration of KCl (20 mM), and varied concentrations of arachidonate (0, 10, 20, 30, or 40 μM). Standard errors of the mean are indicated (n = 20). The p values for the difference in insulin secretion compared to the control were 0.045 at 10 μM arachidonate, 0.009 at 20 μM arachidonate, 0.001 at 30 μM arachidonate, and 0.002 at 40 μM arachidonate.

chidonate concentration in this range actually occur in glucose-stimulated islets.

Discussion

These observations indicate that concentrations of glucose sufficient to stimulate insulin secretion induce a relatively selective accumulation of free arachidonic acid in isolated pancreatic islets and that the amount of arachidonate that accumulates is sufficient to achieve a concentration increment of 38–75 μM. Most of this arachidonate remains within the islet, and little is released into the extracellular medium. Glucose-induced hydrolysis of arachidonate from islet membrane phospholipid is reflected by the rapid release of the oxygenated arachidonate metabolite PGE_2 into the extracellular medium. The depolarizing secretagogue tolbutamide and the ionophore A23187 also induce arachidonate accumulation within and PGE_2 release from islets, and both of these agents increase the islet cytosolic [Ca^{2+}] by inducing Ca^{2+} influx from the extracellular space (Sturgess et al., 1985; Trube et al., 1986; Rorsman et al., 1984; Hermans & Henquin, 1989). Although glucose is also known to induce Ca^{2+} influx into islet (Rorsman et al., 1984; Arkhammar et al., 1987; Gylfe et al., 1988a,b; Nilsson et al., 1988), glucose was able to induce eicosanoid release from islets even under conditions where Ca^{2+} influx was prevented, including removal of Ca^{2+} from the extracellular medium and addition of EGTA or incubation with the Ca^{2+} channel blockers nifedipine and verapamil. This indicates that a substantial component of glucose-induced arachidonate release is independent of Ca^{2+} influx and must be mediated by some other signaling mechanism. Some phospholipid hydrolytic events induced by glucose in pancreatic islets are known to be triggered by Ca^{2+} influx. For example, glucose induces the rapid accumulation of inositol phosphates in islets, and this phenomenon is largely prevented by the removal of extracellular Ca^{2+} or by the inclusion of verapamil, at concentrations similar to those employed here, in the incubation medium (Biden et al., 1987). The findings reported here indicate that all classes of phospholipid hydrolytic events induced by glucose in islets (in particular, glucose-induced eicosanoid release) cannot be attributed solely to Ca^{2+} influx.

The mechanism by which glucose does in fact trigger release of free arachidonate from islet glycerolipids must be the subject of subsequent investigations. Among the possibilities that must be considered is G-protein-induced activation of phospholipase C and phospholipase A_2 enzymes, as has been suggested in other systems (Fain et al., 1988; Banga et al., 1988; Burch & Axelrod, 1987). Islets are known to contain G-proteins, and G-protein-modifying bacterial toxins exert a striking influence on insulin secretion (Dunlop & Larkins, 1983; Nilsson et al., 1989; Terashima et al., 1987; Laychock & Bilgin, 1987). Before interaction with effector targets such as phospholipases or adenyl cyclase, G-proteins generally undergo an activation process from interaction with a plasma membrane receptor which is occupied by a specific agonist (Gilman, 1987). Glucose is not generally thought to interact with islets via a plasma membrane receptor, however, but rather appears to induce insulin secretion as a consequence of its metabolism within the islet (Malaisse et al., 1979; Hedeskov, 1980; Ashcroft, 1980; Wollheim & Scharp, 1981; Meglasson & Matschinsky, 1986; Prentki & Matschinsky, 1987). It is conceivable that glucose transport proteins (Ashcroft & Stubbs, 1987; Permutt et al., 1988; Mueckler, 1990) within the islet plasma membrane might interact with G-proteins or with phospholipase enzymes directly. A metabolite of glucose might also be imagined to interact with a phospholipase in a direct allosteric manner or indirectly via information-transducing elements such as receptors and G-proteins. Similar roles might be postulated for other products of glucose metabolism, such as ATP. In addition, phosphorylation of phospholipases by regulatory protein kinases activated by one of the second messengers generated in glucose-stimulated islets (e.g., cyclic AMP) might result in phospholipase activation by a mechanism independent of Ca^{2+} influx.

A current schema of the early events that occur within the glucose-stimulated β cell and which participate in the induction

of insulin secretion is that glucose induces the closure of a plasma membrane ATP-sensitive K^+ channel, which accounts for the bulk of the permeability of the resting membrane to K^+ (Cook & Hales, 1984; Ashcroft et al., 1984; Rorsman & Trube, 1985; Cook et al., 1988). Since the resting membrane potential in islets is largely a K^+ potential, the reduction of the permeability of the plasma membrane to K^+ resulting from closure of this channel causes the membrane potential to rise from its negative resting value toward zero. Voltage-sensitive Ca^{2+} channels that are inactive at the resting potential become active as the membrane potential rises, and these channels carry Ca^{2+} into the cell down its concentration gradient, resulting in a rise in the cytosolic [Ca^{2+}] (Rorsman et al., 1984; Arkhammar et al., 1987; Gylfe, 1988a,b; Nilsson et al., 1988). All of the factors requlating these ionic events are not clearly established. For example, although the islet K^+ channel is clearly sensitive to ATP and the metabolism of glucose clearly generates ATP within the islet, the ATP levels within the resting islet appear to be orders of magnitude above that required to induce closure of the K^+ channel (Cook et al., 1988), and there is little demonstrable change in ATP concentration in the glucose-stimulated islet (Meglasson et al., 1988). This suggests that amplification mechanisms which magnify the effects of very small changes in ATP levels or of membrane potential might be involved in producing the ionic and secretory responses to glucose stimulation. The facts that glucose-induced arachidonate release occurs rapidly and occurs in the absence of Ca^{2+} influx raise the possibility that arachidonate itself might participate in the regulation of Ca^{2+} influx within the islet.

The possibility that free arachidonic acid might facilitate voltage-dependent Ca^{2+} entry in islet β cells was suggested by recently reported observations from voltage clamp studies in a clonal pituitary tumor GH_3 cell line that arachidonate affects a shift in the voltage–Ca^{2+} current response relationship toward lower membrane potentials (Vacher et al., 1989). This results in the development of a measurable inward Ca^{2+} current at membrane potentials below those otherwise required to induce activation of voltage-dependent Ca^{2+} channels. The maximal Ca^{2+} current is also achieved at a lower membrane potential in the presence of arachidonate, although the amplitude of the maximal current is unchanged (Vacher et al., 1989). It was reasoned that, if such an effect occurred in islets, arachidonate should augment Ca^{2+} entry and, presumably, insulin secretion at submaximally depolarizing concentrations of K^+. The observed result, that arachidonate shifts the K^+–insulin secretion response relationship to the left on the [K^+] axis (Figure 5), is consistent with the possibility that arachidonate amplifies depolarization-induced Ca^{2+} entry into β cells.

It is unlikely that amplification of K^+-induced insulin secretion merely reflects a detergent effect of arachidonate on islet membranes for the following reasons: (1) Neither basal insulin secretion nor the highest level of K^+-stimulated secretion is affected by arachidonate (Figure 5). (2) The effect of arachidonate plateaus in the concentration range 30–40 μM (Figure 6). (3) Arachidonate concentrations in the range 30–40 μM have been demonstrated not to influence measured islet intracellular volume (Wolf et al., 1986), a parameter which increases dramatically when the plasma membrane is permeabilized by agents such as digitonin. (4) Stimulation of islets with physiologic concentrations of glucose induces an increment in the free arachidonate mass in islets sufficient to raise the cellular concentration by 38–75 μM (Figure 1). (5) Release of macromolecules, such as the enzyme lactate dehydrogenase, from platelets induced by arachidonate requires concentrations of 200 μM and does not occur at 20 μM (Turk & Needleman, 1983), although the latter concentration is effective in amplifying K^+-induced insulin secretion (Figures 5 and 6). (6) Polyunsaturated fatty acids induce release of ^{51}Cr from prelabeled lymphocytes only at concentrations exceeding 80 μM and not at concentrations of 40 μM or below (Chow & Jondal 1990). (7) Fatty acids in water do not form a micellar phase below pH 9 and are therefore unlikely to exert detergent effects on cellular membranes at physiologic pH (Cristola et al., 1988).

Our studies do not establish whether the amplification of depolarization-induced insulin secretion from islets by arachidonate is a direct effect on the fatty acid or whether arachidonate metabolites participate in this response. The islet cyclooxygenase product PGE_2 is thought to exert a negative modulatory effect on insulin secretion (Robertson et al., 1974, 1987; Robertson & Chen, 1977; Robertson, 1986, 1988) by lowering β-cell cyclic AMP levels (Robertson et al., 1987). The release of PGE_2 from secretagogue-stimulated islets in the studies described here is taken only as a marker of hydrolysis of arachidonate from islet membrane phospholipids and is not interpreted as reflecting a pro-secretory role for PGE_2 in islets. In contrast, arachidonate 12-lipoxygenase products are thought to promote insulin secretion from islets (Metz et al., 1983; Metz, 1985, 1988b), and the participation of such compounds in the amplification of depolarization-induced insulin secretion cannot be excluded. The fact that much larger amounts of arachidonate itself than of arachidonate metabolites accumulate in secretagogue-stimulated pancreatic islets (Turk et al., 1984, 1985; Wolf et al., 1986) and in pancreatic exocrine tissue (Hokin, 1985; Chaudhry et al., 1987) has been taken to suggest that arachidonate may have some primary mediator roles in signal transduction that do not require its conversion to metabolites (Hokin, 1985). The facts that inhibitors of arachidonate metabolism do not block the calcium-mobilizing effects of arachidonate in islets (Wolf et al., 1986; Metz et al., 1987) and in other cells (Chow & Jondal, 1990) and that similar effects are exerted by other long-chain fatty acids are compatible with this hypothesis, as is the observation that arachidonate-induced insulin secretion is unaffected by inhibitors of arachidonate oxygenation (Metz et al., 1987) and is mimicked by other long-chain unsaturated fatty acids (Metz, 1988a).

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