Transient Activation of Calcium-Dependent Phospholipase A_2 by Insulin Secretagogues in Isolated Pancreatic Islets†

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ABSTRACT: Arachidonic acid is believed to be an important and necessary mediator of insulin secretion by β-cells of islets of Langerhans, and it may regulate intracellular Ca^{2+} homeostasis. Insulin secretagogues, such as glucose and the muscarinic agonist carbachol, stimulate arachidonic acid accumulation, although the mechanisms involved are controversial: carbachol is believed to stimulate phospholipase A_2, while glucose-induced arachidonic acid release is the result of diacylglycerol hydrolysis [Konrad, R. J., et al. (1992) *Biochem. J. 287*, 283–290]. In insulin-secreting clonal β-cells RINm5F, HIT-T15, and β-TC3, Ca^{2+}-independent phospholipase A_2 was mainly cytosolic, while in islets it was equally distributed between a crude membrane fraction and the cytosol. A membrane-associated Ca^{2+}-dependent phospholipase A_2 was found to be stimulated by millimolar Ca^{2+} concentrations, while a cytosolic Ca^{2+}-dependent activity was activated by micromolar Ca^{2+} levels. In order to determine whether phospholipase A_2 was activated in insulin secretion, we assessed whether pretreatment of intact islets with secretagogues affected phospholipase A_2 activity, which was subsequently measured in membrane or cytosolic fractions. The combination of glucose and carbachol transiently activated Ca^{2+}-dependent (but not Ca^{2+}-independent) phospholipase A_2 activity at 10 min, which corresponded to the peak of arachidonic acid release. No effect was seen with either agonist alone. Our results indicate that activation of Ca^{2+}-dependent phospholipase A_2 may be due to agonist-induced increases in intracellular Ca^{2+}. We suggest that activation of islet Ca^{2+}-dependent phospholipase A_2 may be important in a distal process of insulin secretion, such as secretory granule exocytosis.

The mechanisms involved in insulin secretion by the β-cell of islets of Langerhans are not well understood (MacDonald, 1990; Matschinsky, 1990; Misler et al., 1992; Hedeskov, 1980). Glucose, the major physiological agonist, is an example of a nutrient secretagogue since it has to be metabolized to induce insulin secretion (Malaisse et al., 1979; Meglasson & Matschinsky, 1986). Carbachol, a muscarinic agonist, is a secretagogue which stimulates insulin secretion by binding to a muscarinic receptor at the plasma membrane (Henquin & Nenquin, 1988). Both glucose and carbachol cause insulin exocytosis by increasing intracellular Ca^{2+} levels (Garcia et al., 1988; Rajan et al., 1990; Regazzi et al., 1990; Turk et al., 1987): glucose stimulates voltage-dependent Ca^{2+} entry, while carbachol activates phospholipases C and A_2, which generate the second messengers inositol 1,4,5-triphosphate and arachidonic acid (Turk et al., 1986b, 1987; Konrad et al., 1992b; Wolf et al., 1988b).

Arachidonic acid has recently emerged as an important mediator of insulin secretion (Metz, 1988a, 1991; Turk et al., 1990; Wolf et al., 1993; Jones & Persaud, 1993). Glucose as well as carbachol causes a rapid and significant increase in endogenous arachidonic acid levels as measured by various techniques, including gas chromatography–mass spectrometry (Wolf et al., 1986, 1991; Konrad et al., 1992b, 1993). Inhibition of agonist-induced arachidonic acid accumulation clearly inhibits insulin secretion (Konrad et al., 1992a; Ramanadham et al., 1993b). Several intracellular mechanisms have been described whereby arachidonic acid may control insulin exocytosis (Band et al., 1993). Arachidonic acid has been shown to mobilize Ca^{2+} from the endoplasmic reticulum, resulting in an increase in intracellular Ca^{2+} (Wolf et al., 1986, 1988a; Metz, 1988c; Metz et al., 1987). Arachidonic acid is also believed to facilitate voltage-dependent Ca^{2+} entry into the β-cell (Wolf et al., 1991; Ramanadham et al., 1992). Arachidonic acid may also activate protein kinase C (Metz, 1988b; Shinomura et al., 1991; Basudev et al., 1993). Finally, arachidonic acid in the β-cell is predominantly metabolized to 12-hydroxy-5,8,10,14-eicosatetraenoic acid and prostaglandin E_2, which also modulate insulin secretion (Robertson, 1988; Metz, 1991; Turk et al., 1987; Laychock, 1990; Needleman et al., 1986). Taken together, these studies strongly suggest that a stimulus-induced increase in intracellular arachidonic acid levels is necessary for insulin secretion.

The pathways of agonist-induced arachidonic acid accumulation in islets are controversial (Turk et al., 1993; Wolf et al., 1993). There are two major known pathways for arachidonic acid release in islets. (1) Phospholipase A_2 hydrolysis of membrane phospholipids liberates arachidonic acid from the *sn*-2 position (Glaser et al., 1993; Mayer & Marshall, 1993). (2) Phospholipase C hydrolysis of polyphosphoinositides generates 1,2-diacyl-*sn*-glycerol (usually enriched in arachidonic acid), which in turn may be hydrolyzed by lipases to unesterified arachidonic acid (Dennis et al., 1991; Olson et al., 1991). The demonstration of agonist-induced stimulation of phospholipase A_2 requires proof that its end-products, unesterified fatty acid and lysophospholipid, accumulate intracellularly. We have recently shown that the muscarinic agonist carbachol stimulates islet phospholipase A_2 as demonstrated by the rapid accumulation of lysophos-

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phatidylcholine (Konrad et al., 1992b). Glucose, however, does not result in lysophospholipid accumulation (Konrad et al., 1992b; Turk et al., 1986a). Furthermore, inhibition of diacylglycerol hydrolysis fully abolishes glucose-induced arachidonic acid release but only 2/3 of carbachol-induced arachidonic acid accumulation (Konrad et al., 1992b). These observations suggest that carbachol stimulates phospholipase A_2 and diacylglycerol hydrolysis to generate unesterified arachidonic acid, while glucose-induced arachidonic acid accumulation is due to diacylglycerol hydrolysis (Konrad et al., 1992b; Wolf et al., 1993). Other groups, however, have shown that glucose may stimulate a Ca^{2+}-independent phospholipase A_2 (Laychock, 1982; Gross et al., 1993; Turk et al., 1993).

The calcium dependency of agonist stimulation of arachidonic acid release is also unclear. In studies where unesterified arachidonic acid is measured, the great majority of glucose- or carbachol-induced increase in arachidonic acid is Ca^{2+}-dependent with a minor Ca^{2+}-independent component (Konrad et al., 1992b). Prostaglandin E_2 is the major cyclooxygenase metabolite of arachidonic acid in islets. Endogenous prostaglandin E_2 levels are approximately 2–3 orders of magnitude less than endogenous levels of unesterified arachidonic acid (Turk et al., 1987). Nevertheless, if prostaglandin E_2 is taken as an index of arachidonate release, the agonist-induced Ca^{2+}-independent component appears much larger (Ramanadham et al., 1993b).

We have investigated these issues by measuring phospholipase A_2 activities in islets and in insulin-secreting cells and determining the mechanism of activation of phospholipase A_2 in insulin secretion. We show that secretagogues which stimulate arachidonic acid release transiently stimulate a Ca^{2+}-dependent phospholipase A_2.

EXPERIMENTAL PROCEDURES

Materials

Male virus-free Sprague–Dawley rats (200–250 g) were purchased from Charles-River (Wilmington, MA). Collagenase P was obtained 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). RINm5F cells (passage 8) were a kind gift of Dr. H. Oie (NIH, Bethesda, MD). HIT-T15 cells (passage 60) were purchased from American Type Culture Collection (Rockville, MD). 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, bovine albumin (RIA grade, fraction V). Phospholipid standards were obtained from Serdary Research Laboratories (Port Huron, MI) or Avanti Polar Lipids (Pelham, AL). Neutral lipids and fatty acids were purchased from Nu Chek Prep (Elysian, MN). Organic solvents (HPLC grade or Optima grade) were provided by Fisher Scientific Co. (Pittsburgh, PA). Other chemicals (except as indicated) were purchased from Sigma or Fisher. 1-Stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonyl-*sn*-glycero-3-phosphocholine (135 Ci/mmol) was purchased from Amersham Corporation (Arlington Heights, IL). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (233 Ci/mmol) and 1-palmitoyl-2-[9,10-^{3}H]palmitoyl-*sn*-glycero-3-phosphocholine (33 Ci/mmol) were obtained from New England Nuclear (Wilmington, DE). The liquid scintillation cocktail Universol was purchased from ICN Biomedicals (Costa Mesa, CA).

Methods

Isolation of Islets. In a typical experiment, islets were isolated aseptically from 4–8 male Sprague–Dawley rats. In brief, the bile duct was cannulated, and the pancreas was inflated with approximately 20 mL of Hanks' balanced salt solution supplemented with penicillin (25 units/mL) and streptomycin (25 μg/mL). The distended pancreas was then excised. Lymph nodes, fat, blood vessels, and bile duct were removed under a stereomicroscope. The tissue was then chopped, rinsed extensively with Hanks' solution, and then digested with collagenase P (3–6 mg/mL of tissue) at 39 °C for 6.5 min. The digested tissue was then rinsed with Hanks' solution and then purified by centrifugation on a discontinuous Ficoll gradient [dialyzed and lyophilized; 4 layers of 27%, 23%, 20.5%, and 11% in Hanks' Hepes (25 mM) buffer]. The majority of the islets rose to the 11–20.5% interface. Islets were harvested and washed in "complete" CMRL-1066 culture medium (supplemented with 10% heat-inactivated newborn bovine serum, 2 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin, and containing 5.5 mM D-glucose). This procedure typically provided 350–400 islets per rat, which were then used as described below (Lacy & Kostianovsky, 1967; McDaniel et al., 1983; Wolf et al., 1990).

Insulin-Secreting Cell Line Cultures. Insulin-secreting cells were cultured in T75 or T150 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 (Wollheim et al., 1990). For HIT-T15 cells, the medium was also supplemented with 0.1 μM selenous acid. Media were changed twice weekly, and cells were trypsinized and subcloned weekly. The day prior to the experiment, cells systematically received fresh media. The insulin secretory capacity of these cells was monitored regularly by measuring insulin release in response to appropriate secretagogues (glyceraldehyde for RINm5F, glucose for HIT-T15, and glucose or glucose + carbachol for β-TC3). Cells were used for 30 passages.

Labeling of Fresh Islets with [^{3}H]Arachidonic Acid and Perifusion Studies. Freshly isolated islets were randomly counted into silanized borosilicate 13 × 100 mm tubes (200 islets/tube). Each tube was then incubated for 90 min at 37 °C in an atmosphere of 95% O_2/5% CO_2 with [^{3}H]arachidonic acid (1 μCi in 25 μL of Krebs–Hepes[1] medium (25 mM Hepes, pH 7.4, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% bovine serum albumin) supplemented with 3 mM D-glucose. Islets were washed 5 times with Krebs–Hepes medium (1 mL) prior to use. [^{3}H]-Arachidonic acid-labeled islets (200 per condition) were placed onto a mixed cellulose acetate and nitrate filter (type SM, 5-μm pore size, Millipore Corporation, Bedford, MA) in a Swinnex 13-mm perifusion chamber (Millipore) and allowed to equilibrate at a flow rate of 1 mL/min for half an hour at 37 °C in Krebs–Hepes medium supplemented with 3 mM glucose which was continually gassed with 95% O_2/5% CO_2. The medium was then changed to the experimental condition, and perifusion was pursued for another 30 min at a flow rate of 1 mL/min. The perifusate was collected every 1 or 2 min in borosilicate tubes on ice; its insulin content was assayed by radioimmunoassay, and its radioactivity content was deter-

[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GAP, GTPase-activating protein; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]amino]ethanesulfonic acid.

mined by liquid scintillation counting using the scintillation cocktail Universol. At the end of the experiment, the filter containing the islets was retrieved and its radioactivity content was determined. The dead space in the perifusion system was 2.25 mL; data was corrected accordingly. Results are presented as the fractional efflux of radiolabeled arachidonic acid expressed as the percent release of total radioactivity incorporated in 200 islets per 1-min fraction (Wolf et al., 1989; Lacy et al., 1972).

Islet Incubation. Islets (500–1000) were 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 then removed from each tube and replaced with 1 mL of fresh Krebs–Hepes medium supplemented with 3 mM glucose, 28 mM glucose, 0.5 mM carbachol and 3 mM glucose, or 28 mM glucose and 0.5 mM carbachol. The supernatant was then removed for insulin determination.

Phospholipase A_2 Assay. Islets or insulin-secreting cells (200–500 μg of protein) were rinsed in 106 mM TES, pH 7.4, and 0.1 mM EGTA on ice and then homogenized in 50–200 μL of 106 mM TES, pH 7.4, 0.1 mM EGTA, and 30% glycerol (v/v). In some experiments, the islet homogenates were centrifuged for 5 min at 150000*g* in a Beckman Airfuge to obtain membranes and cytosolic fractions. Phospholipase A_2 activities were measured as described (Hazen et al., 1991). In brief, 85 μL of 106 mM TES, pH 7.4, and 53% glycerol, ±2.4 mM $CaCl_2$, ±2.4 mM $MgCl_2$, and ±1.17 mM EGTA, was dispensed into silanized 13 × 100 mm round-bottom, screw-cap borosilicate tubes. The enzyme source was then added (10 μL), and the reaction was started by the addition of 0.1 μCi of the radioactive substrate [final reaction concentration: 2 μM of 1-stearoyl-2-[^{3}H]arachidonyl-*sn*-glycero-3-phosphocholine (500 Ci/mol) in 5% ethanol]. In some experiments, 1-palmitoyl-2-[^{3}H]palmitoyl-*sn*-glycero-3-phosphocholine was also used, without any difference in apparent specific activity of islet phospholipase A_2. Tubes were sonicated in a water bath and then incubated at 37 °C with shaking in a Dubnoff 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 mg/mL butylated hydroxytoluene, and the tubes were plunged for 15 min into a dry ice/alcohol bath. Prior to extraction, carrier amounts (5 μg) of phosphatidylcholine and fatty acid were added to each tube to aid in recovery, followed by 1 mL of chloroform and 1 mL of water. Tubes were vortexed (1 min), sonicated (30 min, 4 °C), and vortexed (1 min). Tubes were centrifuged in a refrigerated tabletop centrifuge (15 min, 4 °C, 800*g*). The lower organic phase was then transferred with a silanized Pasteur pipette to a clean silanized 13 × 100 mm conical 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 then washed twice with water (3 mL), concentrated twice under nitrogen in a TurboVap evaporator (Zymark, Hopkinton, MA), and reconstituted in 25 μL of chloroform. With this extraction procedure, recovery of [^{3}H]arachidonic acid was 95.6% (n = 3). Samples were spotted onto the preadsorbent zone of channeled silica gel G TLC 20 × 20 cm plates (Analtech, Newark, DE) which had been activated for 30 min at 110 °C. Plates were developed for 30–45 min in petroleum ether (30–60 °C)/diethyl ether/acetic acid (140:60:2, v/v/v) (Wolf et al., 1986). The radioactivity of the chromatogram was quantitated with a Berthold 284 linear analyzer (Wallac Inc., Gaithersburg, MD) 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 60 min. The instrument detected radioactive peaks as small as 50–100 dpm with a resolution of 0.5 mm. Data analysis was performed using version 7.19 of the Berthold 1D-TLC software. Radioactive peaks corresponding to free arachidonic acid and phosphatidylcholine were integrated. Peak identity was assigned by comparison with iodine-stained cold standards and radiolabeled commercial [^{3}H]arachidonic acid. Typically, the following R_f were obtained: phospholipids, 0; monoacylglycerol, 0.19; diacylglycerol, 0.45; arachidonic acid, 0.63; triacylglycerol, 0.88. To calculate phospholipase A_2 activity, the integrated counts in the arachidonic acid region (after the counts contributed from the blank phospholipid were subtracted) were expressed as a percentage of total radioactivity on the plate, converted to picomoles of phospholipid hydrolyzed by multiplying by 200 pmol (the mass of phospholipid originally present in each reaction tube), and normalized to the mass of protein. Phospholipase A_2 activity was expressed as apparent specific activity (picomoles of phosphatidylcholine hydrolyzed/milligram of protein).

In some experiments, the hydrolysis products of 1-stearoyl-2-[^{3}H]arachidonyl-*sn*-glycero-3-phosphocholine were analyzed by 2D-TLC. In brief, samples were applied to 10 × 10 cm high-performance HP-K silica gel TLC plates (Whatman Biosystems Inc., Clifton, NJ) which had been activated for 30 min at 110 °C. Plates were developed in the first dimension with chloroform/methanol/ammonium hydroxide (65:35:5.5, v/v) for 30 min. Plates were carefully dried (60 min) and then developed in the second dimension with chloroform/methanol/formic acid/water (55:28:5:1, v/v) for 30 min (Thomas & Holub, 1991; Mitchell et al., 1986). Radioactivity of each plate was quantitated by two-dimensional analysis with the Berthold linear analyzer which was fitted with a 2-mm entrance window. Each plate was scanned for at least 12 h. Resolution was 0.5 mm in the first dimension and 2 mm in the second dimension. Data analysis was performed using version 4.07 of the Berthold 2D-TLC software (Konrad et al., 1991).

Other Methods. Free calcium concentrations were maintained by the addition of EGTA and were calculated as previously described in detail (Wolf et al., 1986). Free calcium concentrations were systematically checked with a calibrated Orion calcium electrode as described (Wolf et al., 1986). Protein determinations were performed in microtiter plates using the bicinchoninic acid assay with bovine serum albumin as standard (Smith et al., 1985). Phosphatidylcholine stocks were calibrated by measuring their ester content (Kates, 1986). Insulin radioimmunoassay was performed by the University of Pennsylvania Diabetes Endocrine Research Center.

Data Analysis. Results are expressed as the mean ± SE. Statistical analysis was performed using version 5.0 of Statgraphics (STSC Inc., Rockville, MD). Data were analyzed by one-way or two-way analysis of variance as appropriate followed by multiple comparisons between means using the least significant difference test. A probability of $p < 0.05$ was considered statistically significant.

RESULTS

Insulin Secretagogue Stimulation of Arachidonic Acid Release by Islets. Phospholipase A_2 activity was first measured indirectly by labeling fresh isolated pancreatic islets for 90 min with [^{3}H]arachidonic acid. Labeled islets were then placed in a perifusion chamber, which allows the

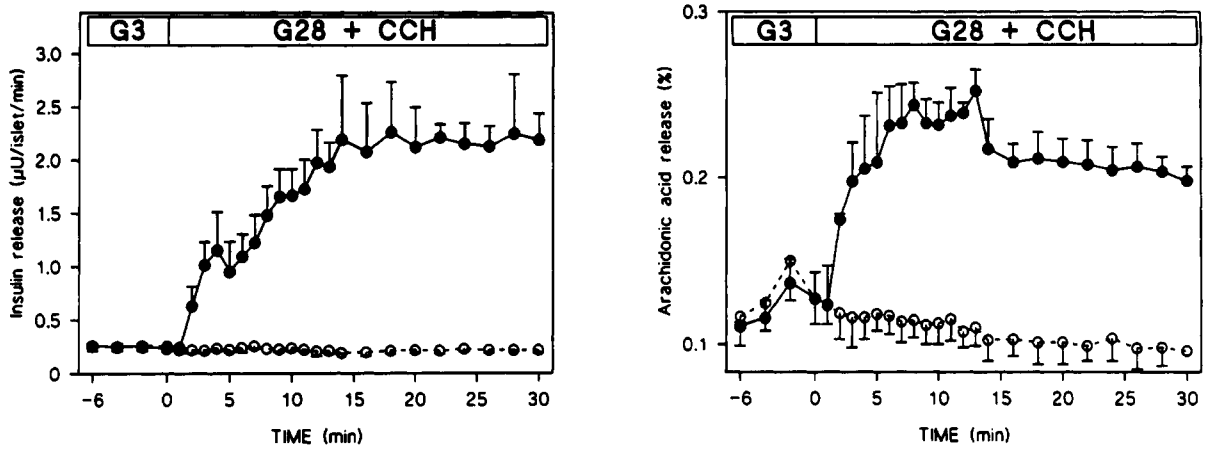

FIGURE 1: Time course of the effect of insulin secretagogues on insulin secretion and [^{3}H]arachidonic acid release from perifused islets prelabeled with [^{3}H]arachidonic acid. Isolated pancreatic islets (200 per condition) were prelabeled for 90 min at 37 °C in 50 μL of Krebs–Hepes medium (25 mM Hepes, pH 7.4, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% BSA) supplemented with 3 mM glucose and [^{3}H]arachidonic acid (4 μCi). Labeled islets were then preincubated for 30 min in a perifusion chamber (flow rate of 1 mL/min) at 37 °C, incubated for another 30 min in Krebs–Hepes medium supplemented with 3 mM glucose (○) or 28 mM glucose + 0.5 mM carbachol (●), and gassed with 95% O_2/5% CO_2. Perifusate was collected every 1 min and assayed for its insulin and [^{3}H]arachidonic acid content. Results are shown as the mean ± SE and are expressed as microunits of insulin/islet/min (left panel) or fractional release of [^{3}H]arachidonic acid (right panel). Total [^{3}H]arachidonic acid incorporation into 200 islets was 295 714 ± 27 804 cpm (n = 4). Similar results were obtained in two other experiments.

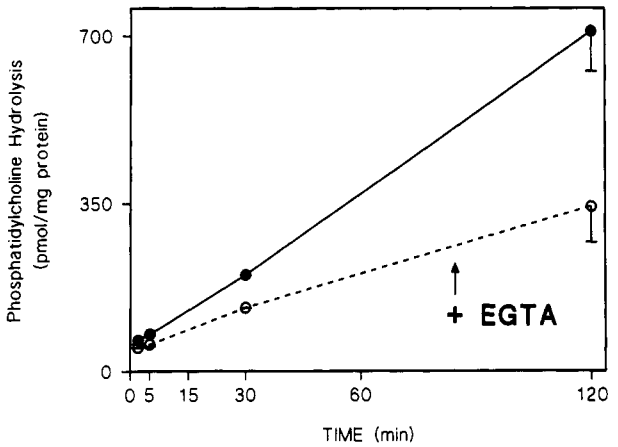

FIGURE 2: Time course of phosphatidylcholine hydrolysis in islet homogenates. Phospholipase A_2 activity was measured in islet homogenates as described in Experimental Procedures in the presence of 2 mM $CaCl_2$ (●) or 1 mM EGTA (○). Results are shown as the mean ± SE from 4–7 observations per time point and are expressed as picomoles of phosphatidylcholine hydrolyzed/milligram of islet protein.

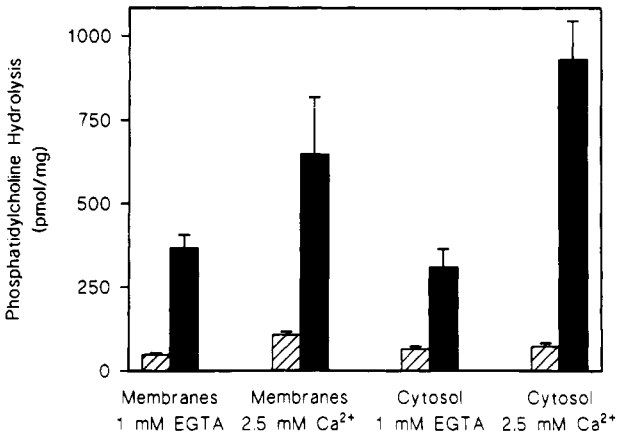

FIGURE 3: Effect of glycerol on islet phospholipase A_2 activity in membrane and cytosolic fractions. Isolated islets were homogenized and centrifuged at 150000g to obtain crude membrane and cytosol fractions. Phospholipase A_2 activity was measured as described in Experimental Procedures in the absence (hatched bars) or presence (solid bars) of 50% glycerol. Results are shown as the mean ± SE from 4–10 observations per condition and are expressed as picomoles of phosphatidylcholine hydrolyzed/milligram of islet protein.

continuous and simultaneous monitoring of insulin secretion and [^{3}H]arachidonic acid release. Under these conditions, the combination of stimulatory concentrations of glucose (28 mM) and of the muscarinic agonist carbachol (0.5 mM) caused a nearly 6-fold increase in insulin release at 4 min and a nearly 12-fold increase by 14 min (Figure 1). The dynamic release of [^{3}H]arachidonic acid was also determined in parallel. Glucose and carbachol rapidly stimulated [^{3}H]arachidonic acid release, which peaked by 10 min (Figure 1). Since [^{3}H]-arachidonic acid release by perifused islets is a reflection of various intracellular events which include phospholipase activation, reacylation of arachidonic acid into phospholipids, and intracellular arachidonic acid metabolism, we then measured phospholipase A_2 directly.

Characterization and Localization of Phospholipase A_2 Activities in Insulin-Secreting β-Cells. In the next series of experiments, phospholipase A_2 activity in islets and insulin-secreting β-cells was measured as the hydrolysis of exogenous 1-stearoyl-2-[^{3}H]arachidonyl-*sn*-glycero-3-phosphocholine to [^{3}H]arachidonic acid. As shown in Figure 2, both calcium-dependent and -independent phospholipase A_2 activities were measured in islet homogenates. Under these experimental conditions, no hydrolysis product of phosphatidylcholine other than arachidonic acid was detected (as measured by 2D-TLC), indicating that the assay was specific for phospholipase A_2. Approximately 50% of the phospholipase A_2 activity was calcium-independent. Glycerol has been reported to stabilize phospholipase A_2 activities in adrenal chromaffin cells (Hildebrandt & Albanesi, 1991). Glycerol significantly increased the specific activity of phospholipase A_2 in membranes and cytosolic fractions prepared from islet homogenates (Figure 3).

Since islets of Langerhans are composed of approximately 85% insulin-secreting β-cells as well as glucagon- and somatostatin-secreting cells, phospholipase A_2 activity was also measured in various clonal insulin-secreting β-cells (Table I). RINm5F cells are derived from a radiation-induced insulinoma in rats and secrete insulin in response to the nutrient glyceraldehyde and the muscarinic agonist carbachol (Wollheim et al., 1990). HIT-T15 clones are obtained after simian virus 40 (SV40) infection and transformation of Syrian

Table I: Localization of Phospholipase A_2 Activity to Insulin-Secreting β-Cells[a]

condition	phospholipase A_2 activity (pmol/mg)	
	Ca^{2+}-independent	total
RINm5F (trypsinized)		
membranes	98.0 ± 10.0	48.5 ● 9.5
membranes + glycerol	80.5 ± 8.5	66.0 ● 10.0
cytosol	176.0 ± 14.0	145.5 ± 52.5
cytosol + glycerol	42.0	64.5 ± 7.5
HIT-T15 (trypsinized)		
homogenate	40.3 ± 2.4	29.0 ± 3.1
homogenate + glycerol	130.3 ± 6.1	104.7 ± 8.8
RINm5F (scraped + glycerol)		
membranes	68.3 ± 8.5	128.6 ± 17.0
cytosol	389.1 ● 51.1	120.9 ± 42.7
HIT-T15 (scraped + glycerol)		
membranes	40.0 ± 5.1	53.5 ± 4.0
cytosol	81.5 ± 13.2	103.3 ± 6.5
β-TC3 (scraped + glycerol)		
membranes	22.0 ± 0.7	35.3 ± 6.3
cytosol	45.5 ± 1.9	53.8 ± 2.7

[a] Insulin-secreting clonal β-cells were either trypsinized or scraped with a rubber policeman. Homogenates and subcellular fractions were prepared as described in Experimental Procedures and used as the source of phospholipase A_2. Phospholipase A_2 was measured in the presence of 2 mM EGTA (for Ca^{2+}-independent activity) or 2 mM $CaCl_2$ (for total activity), ±50% glycerol. Results are shown as the mean ±SE from 2–8 observations. Ca^{2+}-dependent phospholipase A_2 activity may be calculated as total activity − Ca^{2+}-independent activity.

hamster β-cells and respond to glucose (Wollheim et al., 1990). The β-TC3 line is derived from an insulinoma which develops in transgenic mice expressing the SV40 T antigen uniquely in the pancreatic β-cell. β-TC3 cells have a high insulin content and secrete insulin in response to glucose and carbachol (Efrat et al., 1988; D'Ambra et al., 1990).

Trypsinization of β-cells resulted in a significant loss of the calcium-dependent phospholipase A_2 activity but did not affect the calcium-independent phospholipase A_2 activity (Table I). The calcium-independent phospholipase A_2 activity in all three cell types was predominantly cytosolic, while the calcium-dependent phospholipase A_2 activity was equally distributed between cytosol and membranes in HIT-T15 and β-TC3 cells but was predominantly membrane-bound in RINm5F cells (Table I). The pH dependency of phospholipase A_2 is shown in Figure 4. In membranes, the optimum pH of the Ca^{2+}-independent activity was 5, while in the presence of 2 mM Ca^{2+} optimum pH was 8 (Figure 4A). In the cytosol, the Ca^{2+}-independent phospholipase A_2 had a pH optimum between 7.4 and 9, while at pH 5 less than 6% activity remained (Figure 4B). A similar pattern for the cytosolic phospholipase A_2 was observed in the presence of Ca^{2+}.

Activation of Calcium-Dependent Phospholipase A_2 by Insulin Secretagogues in Islets. There is substantial evidence suggesting that phospholipase A_2 may be activated during insulin secretion. In these experiments, islets were incubated in Krebs–Hepes medium supplemented with 3 mM glucose or 28 mM glucose and 0.5 mM carbachol. After incubation, phospholipase A_2 was measured in the membrane and cytosolic fractions. The combination of glucose (28 mM) and carbachol (0.5 mM) caused a significant increase in phospholipase A_2 activity, which was transient (Figures 5 and 6). In the membrane fraction, at 5 min of incubation no secretagogue-induced changes in Ca^{2+}-dependent phospholipase A_2 were observed; at 10 min there was a significant 60% increase in

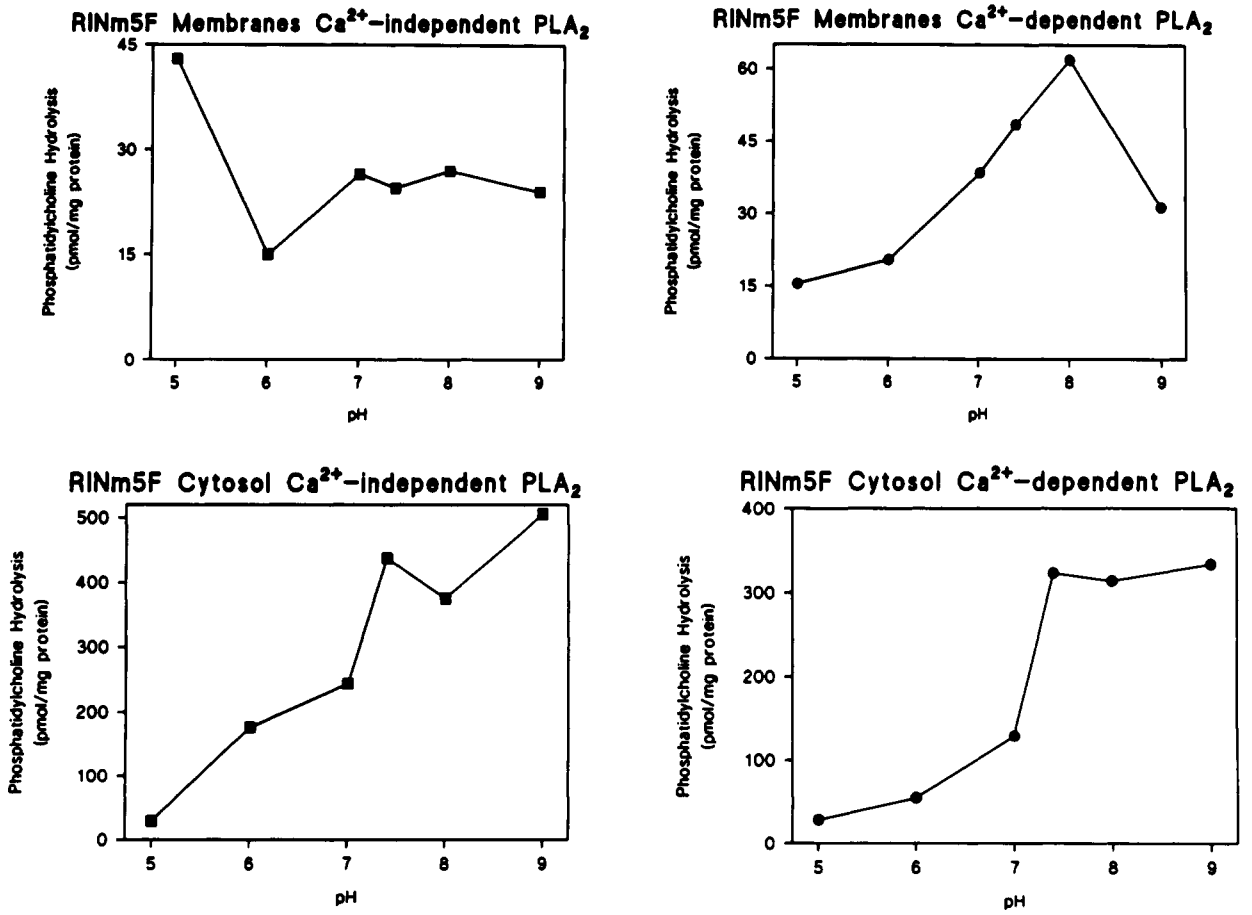

FIGURE 4: (A, top two panels) pH dependency of membrane phospholipase A_2 in RINm5F cells. RINm5F cells were homogenized and centrifuged at 150000g to obtain crude membrane and cytosol fractions. Phospholipase A_2 activity was measured as described in Experimental Procedures. Results are shown as the mean of duplicate observations per condition and are expressed as picomoles of phosphatidylcholine hydrolyzed/milligram of cell protein. (B, bottom two panels) pH dependency of cytosolic phospholipase A_2 in RINm5F cells.

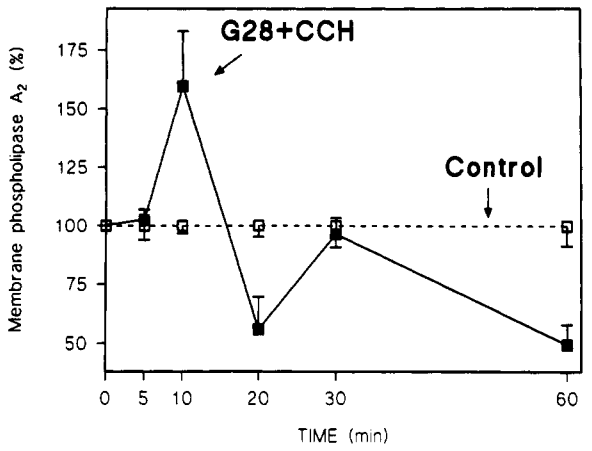

FIGURE 5: Transient activation of membrane calcium-dependent phospholipase A_2 by insulin secretagogues in isolated pancreatic islets. Isolated islets were stimulated with 3 mM glucose in Krebs–Hepes medium (Control: □) or 28 mM glucose and 0.5 mM carbachol (G28 + CCH: ■) for the indicated times. Following stimulation, membranes and cytosolic fractions were prepared at 150000*g* and calcium-dependent phospholipase A_2 activity was measured as described in Experimental Procedures. Due to the day-to-day variation in absolute phospholipase A_2 activity, results are shown as the mean ± SE of membrane phospholipase A_2 activity expressed as a percentage of the same-day 3 mM glucose control from 4–18 observations per time point. Control membrane phospholipase A_2 activity (in picomoles of phosphatidylcholine hydrolyzed/milligram of protein) was 1074 ± 64 at 5 min (n = 4), 804 ± 42 at 10 min (n = 18), 1090 ± 33 at 20 min (n = 6), 851 ± 43 at 30 min (n = 10), and 388 ± 34 at 60 min (n = 8).

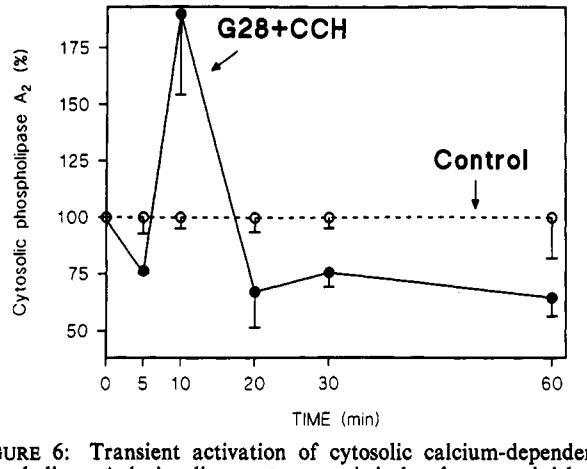

FIGURE 6: Transient activation of cytosolic calcium-dependent phospholipase A_2 by insulin secretagogues in isolated pancreatic islets. Isolated islets were stimulated with 3 mM glucose in Krebs–Hepes medium (Control: ○) or 28 mM glucose and 0.5 mM carbachol (G28 + CCH: ●) for the indicated times. Following stimulation, membrane and cytosol fractions were prepared at 150000*g*, and calcium-dependent phospholipase A_2 activity was measured as described in Experimental Procedures. Due to the day-to-day variation in absolute phospholipase A_2 activity, results are shown as the mean ± SE of cytosolic phospholipase A_2 activity expressed as a percentage of the same-day 3 mM glucose control from 4–17 observations per time point. Control cytosolic phospholipase A_2 activity (in picomoles of phosphatidylcholine hydrolyzed/milligram of protein) was 312 ± 22 at 5 min (n = 4), 738 ± 93 at 10 min (n = 15), 1899 ± 141 at 20 min (n = 9), 453 ± 41 at 30 min (n = 10), and 120 ± 22 at 60 min (n = 8).

activity compared to control (Figure 5). By 20 and 60 min, secretagogue-stimulated phospholipase A_2 activity had decreased by 46% and 50%, respectively. A similar pattern was observed for cytosolic Ca^{2+}-dependent phospholipase A_2 activity (Figure 6). Glucose or carbachol alone, after a 10-min incubation, did not elicit any significant increase in Ca^{2+}-dependent phospholipase A_2 activity (data not shown). Ca^{2+}-independent phospholipase A_2 activity in membranes and cytosol was also not affected by glucose and carbachol (membrane phospholipase A_2 activity, control, 100 ± 2.0%, glucose + carbachol, 96.9 ± 14.7%; cytosol phospholipase A_2 activity, control, 100 ± 6.2%, glucose + carbachol, 93.4 ± 10.6%; n = 14).

Table II: Calcium-Induced Translocation of Membrane Calcium-Dependent Phospholipase A_2 in Isolated Pancreatic Islets[a]

homogenization condition	calcium-independent phospholipase A_2 (pmol/mg)		total phospholipase A_2 (pmol/mg)	
	membranes	cytosol	membranes	cytosol
2 mM EGTA	264.2 ± 44.7	70.0 ± 7.0	436.5 ± 75.0	241.7 ± 32.8
2 mM $CaCl_2$	179.8 ± 23.6	73.2 ± 4.5	608.8 ± 94.9[b]	296.7 ± 82.7

[a] Isolated islets were homogenized in the presence of 2 mM EGTA or 2 mM $CaCl_2$. Membrane and cytosol fractions were prepared as described in Experimental Procedures, and phospholipase A_2 activity was measured in the absence (calcium-independent activity) or presence of calcium [(total activity = Ca^{2+}-independent + Ca^{2+}-dependent activities). Results are shown as the mean of six determinations and are expressed as picomoles of phosphatidylcholine hydrolyzed/milligram of islet protein. Ca^{2+}-dependent phospholipase A_2 activity may be calculated as total activity − Ca^{2+}-independent activity. [b] $p < 0.05$ compared to 2 mM EGTA control.

The calcium dependency of phospholipase A_2 was examined next. Increasing the free Ca^{2+} concentration from nano- to millimolar levels caused a significant increase in the Ca^{2+}-dependent phospholipase A_2 activity associated with membranes without affecting the cytosolic activity (Table II). Membrane-associated phospholipase A_2 activity was stimulated by millimolar levels of Ca^{2+}, while free Ca^{2+} concentrations in the 10^{-7} to 10^{-4} M range had no effect (Figure 7). Cytosolic phospholipase A_2 activity, however, had a very different Ca^{2+}-dependency pattern since it was stimulated by micromolar concentrations of free Ca^{2+} (Figure 7).

DISCUSSION

Our study demonstrates the presence of several phospholipase A_2 activities in islets and insulin-secreting cells. In islets, membrane-associated Ca^{2+}-dependent phospholipase A_2 was stimulated by millimolar Ca^{2+} concentrations, while cytosolic Ca^{2+}-dependent phospholipase A_2 was activated by micromolar Ca^{2+} concentrations. Stimulation of islets with insulin secretagogues caused a transient activation of Ca^{2+}-dependent phospholipase A_2. Ca^{2+}-independent phospholipase A_2 represented approximately one-half of the total phospholipase A_2 activity in islets and was equally distributed between membrane and cytosolic fractions. In insulin-secreting clonal β-cells, however, this activity was predominantly cytosolic. Ca^{2+}-dependent phospholipase A_2 activities were detected in crude membrane and cytosolic fractions. Further purification of this enzyme will be necessary to determine its precise subcellular localization as well as its substrate specificity. Differences between the insulin-secreting β-cell lines and the islets may result from different experimental conditions: islets are freshly isolated from rat pancreas by collagenase digestion and very briefly maintained in CMRL-1066 medium (with 5.5 mM glucose), while the β-cell lines are typically cultured for numerous passages in RPMI-1640 medium containing 11 mM glucose. In addition, after prolonged culture, HIT-T15 and β-TC3 cells lose the capacity to secrete insulin in response to secretagogues (Robertson et al., 1992).

A Ca^{2+}-independent phospholipase A_2 has recently been described in islets which hydrolyzes phospholipids with a vinyl ether linkage at the *sn*-1 position and is selective for arachidonic acid in the *sn*-2 position (Gross et al., 1993). This plasmalogen-selective Ca^{2+}-independent phospholipase A_2 was predominantly membrane-associated in islets and also displayed about 3–6-fold less activity against 1,2-diacyl-*sn*-glycero-3-phos-

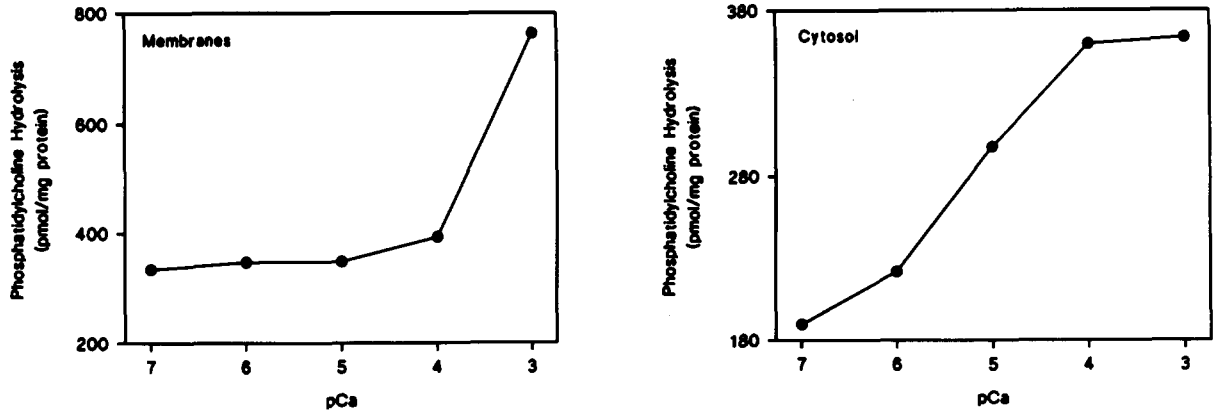

FIGURE 7: Calcium dependency of phospholipase A_2 activity in membrane and cytosol fractions from isolated pancreatic islets. Islet membrane and cytosol fractions were prepared as described in Experimental Procedures, and phospholipase A_2 activity was measured in the presence of increasing calcium concentrations. Results are shown as the mean of phospholipase A_2 activity from quadruplicate determinations and are expressed as picomoles of phosphatidylcholine hydrolyzed/milligram of islet protein (left panel, membranes; right panel, cytosol).

phocholine (Gross et al., 1993). Under our experimental conditions, in which 1,2-diacyl-*sn*-glycero-3-phosphocholine was used as a substrate, we could not detect any activation of Ca^{2+}-independent phospholipase A_2 in islets stimulated with insulin secretagogues. Although these findings are somewhat at variance with a previous report which showed that specific inhibition of islet plasmalogen-selective Ca^{2+}-independent phospholipase A_2 strongly correlated with inhibition of glucose-induced insulin secretion (Ramanadham et al., 1993a,b), they do not exclude a role for Ca^{2+}-independent phospholipase A_2 in the signal transduction of insulin secretion.

There has been indirect evidence suggesting that Ca^{2+}-dependent phospholipase A_2 may be involved in insulin secretion: (1) Secretagogue-induced arachidonic acid release is largely Ca^{2+}-dependent (Dunlop & Larkins, 1984; Wolf et al., 1986, 1991; Metz, 1988a, 1991; Konrad et al., 1992b). (2) Inhibition of secretagogue-induced arachidonic acid accumulation inhibits insulin secretion (Konrad et al., 1992a). (3) Ca^{2+}-dependent phospholipase A_2 is detected in islet homogenates (Laychock, 1982; Schrey & Montague, 1983; Dunlop & Larkins, 1984; Metz, 1987; Konrad et al., 1992a). Our study shows that Ca^{2+}-dependent phospholipase A_2 is localized to insulin-secreting β-cells. There appeared to be two different types of activity: a membrane-associated activity stimulated by millimolar Ca^{2+} with an alkaline pH optimum and a cytosolic phospholipase A_2 activated by micromolar Ca^{2+}. Membrane Ca^{2+}-dependent phospholipase A_2 is most likely a 14-kDa group I secretory phospholipase A_2 since its mRNA has been recently detected in islets (Metz et al., 1991), and Western blot analysis of islet and insulin-secreting β-cell membrane (but not cytosolic) fractions with anti-porcine pancreatic phospholipase A_2 antibody detects a 14-kDa protein[2] (Kim et al., 1989; Bar-Sagi, 1991). Similar findings have been reported in a macrophage-like cell line (Ulevitch et al., 1988), in mast cells (Murakami et al., 1992), and in human platelets and synovial fluid (Johansen et al., 1992).

A high molecular weight cytosolic Ca^{2+}-dependent phospholipase A_2 has been recently purified from human monocytic U937 cells, and it is activated by micromolar Ca^{2+} concentrations (Diez & Mong, 1990; Clark et al., 1990; Kramer et al., 1991; Leslie, 1991). The cDNA encoding this protein has been isolated and expressed: the expressed protein has no homology with secreted forms of phospholipase A_2 and has a 45 amino acid domain with homology to protein kinase C, p65, GAP, and phospholipase C (Clark et al., 1991; Sharp et al., 1991). Cytosolic phospholipase A_2 is phosphorylated and activated by MAP kinase (Lin et al., 1993; Nemenoff et al., 1993). Whether the same cytosolic phospholipase A_2 is present in islets remains to be determined. Preliminary experiments using Western blots of islet and β-TC3 immunoprecipitates with a specific antibody suggest that it may be similar to the high molecular weight cytosolic phospholipase A_2 of U937 cells[3] (Clark et al., 1991).

The role of phospholipase A_2 and arachidonic acid in insulin secretion is controversial (MacDonald, 1990; Metz, 1991; Turk et al., 1993). So far, there has been mainly indirect evidence suggesting that phospholipase A_2 is activated during insulin secretion (Laychock, 1982; Schrey & Montague, 1983; Dunlop & Larkins, 1984; Metz, 1987; Konrad et al., 1992a,b). In a previous report, the combination of glucose and carbachol was found to induce the intracellular accumulation of lysophosphatidylcholine in intact isolated islets, suggesting that phospholipase A_2 is activated (Konrad et al., 1992b). The present study establishes that the Ca^{2+}-dependent phospholipase A_2 is activated during insulin secretion. Activation was not detected before 10 min of stimulation with the combination of glucose and carbachol, which also corresponds to the time of peak arachidonic acid release (see Figure 1). The fact that activation of Ca^{2+}-dependent phospholipase A_2 is not seen earlier suggests that it is probably not involved in the initial steps of glucose recognition by the β-cell. This observation is consistent with our previous report that glucose-induced arachidonic acid release does not involve phospholipase A_2, but is mediated through diacylglycerol hydrolysis (Konrad et al., 1992b).

The mechanism of phospholipase A_2 activation in islets appears to result from an increase in intracellular Ca^{2+}: (1) Millimolar Ca^{2+} increased the association and specific activity of membrane phospholipase A_2. (2) Micromolar Ca^{2+} directly stimulated the cytosolic phospholipase A_2. (3) The pattern of transient activation of phospholipase A_2 by glucose and carbachol is consistent with the effects of these secretagogues on intracellular Ca^{2+} levels in β-cells (Hellman et al., 1992; Theler et al., 1992). In particular, the combination of glucose and carbachol elicits a substantial increase in intracellular Ca^{2+} compared to either agonist alone (Hellman et al., 1992). Furthermore, studies of cytosolic Ca^{2+} in individual β-cells have shown that glucose stimulation enhances not only the amplitude of the carbachol response but also the proportion of responding cells (Theler et al., 1992). These observations most likely explain why neither secretagogue alone caused a detectable increase in Ca^{2+}-dependent phospholipase A_2

[2] Y. Camille Jolly and Bryan A. Wolf, manuscript in preparation.

[3] Y. Camille Jolly and Bryan A. Wolf, unpublished observations.

activation. The transient nature of phospholipase A_2 activation may also be explained by the pattern of intracellular Ca^{2+} in individual β-cells: typically the effect of carbachol and glucose on cytosolic Ca^{2+} is a peak present for the first few minutes, followed by oscillations of a much lower amplitude (Hellman et al., 1992).

There are several alternative mechanisms of phospholipase A_2 activation in islets stimulated with glucose and carbachol which deserve consideration. G-protein regulation of phospholipase A_2 has been demonstrated for α-adrenoreceptors and the rhodopsin system of the rod outer segments in retina (Axelrod, 1990). The muscarinic agonist carbachol is known to be coupled to phospholipase C through a G-protein (Dunlop & Larkins, 1986), but there is no evidence for G-protein coupling to phospholipase A_2. Protein kinases have also been implicated in the regulation of phospholipase A_2 (Glaser et al., 1993). The high molecular weight cytosolic phospholipase A_2 has recently been shown to be a substrate of mitogen-activated protein kinase, a member of the extracellular-signal-regulated kinase (ERK) family (Lin et al., 1993). Full activation of phospholipase A_2 required its phosphorylation on serine residue 505 (Lin et al., 1993). Protein kinase C activation has also been implicated in the activation of phospholipase A_2 in various cell types (Wijkander & Sundler, 1992; Nemenoff et al., 1993; Glaser et al., 1993; Church et al., 1993; Asaoka et al., 1993; Rehfeldt et al., 1993). In particular, purified protein kinase C phosphorylates at least three sites of purified high molecular weight cytosolic phospholipase A_2 (Nemenoff et al., 1993). Since carbachol is known to stimulate diacylglycerol production and activate protein kinase C in islets (Wolf et al., 1989; Easom et al., 1990), while glucose appears to cause translocation of the α isozyme of protein kinase C (Ganesan et al., 1990, 1992), it is possible that the combination of protein kinase C activation and increase in intracellular Ca^{2+} induced by glucose results in phospholipase A_2 activation. Further studies will be needed to elucidate the role of phosphorylation in islet phospholipase A_2 activation.

Activation of Ca^{2+}-dependent phospholipase A_2 in islets clearly results in arachidonic acid release, which is necessary for insulin release (Konrad et al., 1992a; Ramanadham et al., 1993b). Our data does not support a role for Ca^{2+}-dependent phospholipase A_2 in mediating the early or proximal signals involved in agonist recognition by the β-cell. It is tempting to speculate, however, that Ca^{2+}-dependent phospholipase A_2 may have a role in the process of exocytosis of insulin secretory granules. In other secretory cells, such as the adrenal chromaffin cells and the parotid gland, phospholipase A_2 has been localized to the secretory granules (Dunning & Taborsky, 1991; Mizuno et al., 1991; Hildebrandt & Albanesi, 1991), where it may have a role in exocytosis (Creutz, 1992). In particular, in adrenal chromaffin cells, Exo1, a 30-kDa cytosolic protein, has been shown to stimulate exocytosis (Morgan & Burgoyne, 1992). Exo1 is related to a family of proteins called 14-3-3 (Aitken et al., 1992), and it has recently been shown that platelet cytosolic phospholipase A_2 protein belongs to this family of proteins (Zupan et al., 1992), although others have found no evidence of phospholipase A_2 activity in purified 14-3-3 proteins (Morgan et al., 1993). It is possible that a similar mechanism may operate in insulin exocytosis from the β-cell.

In summary, we have shown that islets and insulin-secreting β-cells contain Ca^{2+}-independent and Ca^{2+}-dependent phospholipase A_2 activities. The latter are stimulated by millimolar Ca^{2+} concentrations in membranes and by micromolar Ca^{2+} levels in the cytosol. Furthermore, insulin secretagogues transiently activate Ca^{2+}-dependent phospholipase A_2 activity possibly by increasing intracellular Ca^{2+} concentrations. The pattern of phospholipase A_2 activation suggests that it may be involved in the distal process of insulin exocytosis.

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