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Carbachol stimulation of phospholipase A_2 and insulin secretion in pancreatic islets

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Arachidonic acid has been implicated as a second messenger in insulin secretion by islets of Langerhans. D-Glucose, the major physiological stimulus, increases unesterified arachidonate accumulation in islets. We now show, for the first time, that the muscarinic agonist carbachol, at concentrations which stimulate insulin secretion, causes a rapid and nearly 3-fold increase in arachidonic acid accumulation in islets. The combination of glucose and carbachol has an additive effect on unesterified arachidonate release. There is a large component of secretagogue-induced arachidonate accumulation that is independent of extracellular Ca^{2+}. Carbachol stimulation of arachidonic acid release is mediated by activation of phospholipase A_2, as demonstrated by early increases in endogenous lysophosphatidylcholine. In addition to phospholipase A_2 activation, carbachol-induced arachidonic acid accumulation also appears to involve diacylglycerol hydrolysis, since the diacylglycerol lipase inhibitor RG80267 partly inhibited arachidonic acid accumulation. In contrast, glucose-induced arachidonic acid accumulation appears to reflect diacylglycerol hydrolysis entirely. Our observations indicate that phospholipase A_2 has an important role in muscarinic-induced insulin secretion.

INTRODUCTION

Insulin secretion from the islets of Langerhans can be stimulated by different types of secretagogues (Ashcroft, 1980). D-Glucose, which belongs to the class of fuel secretagogues, is the major physiological stimulus (Malaisse *et al.*, 1979; Hedeskov, 1980). Another class of insulin secretagogues comprises agonists that bind to plasma-membrane receptors of the β-cell (Zawalich & Rasmussen, 1990). For example, the muscarinic agonist carbachol has been widely used to probe the vagal component of insulin secretion (Prentki & Matschinsky, 1987; Turk *et al.*, 1987; Garcia *et al.*, 1988).

The mechanism whereby glucose and other fuel secretagogues stimulate insulin secretion is not completely elucidated (Meglasson & Matschinsky, 1986; Matschinsky, 1990). Glucose oxidation is essential for insulin secretion (Malaisse *et al.*, 1979; Wollheim & Sharp, 1981; Meglasson & Matschinsky, 1986). Inhibition of glucose oxidation clearly inhibits insulin release. The precise details of the mechanism coupling glucose oxidation to insulin secretion, however, are less clear. It is currently believed that oxidation of fuel secretagogues increases intracellular ATP (Rajan *et al.*, 1990), although this view has been challenged by some groups (MacDonald, 1990; Ghosh *et al.*, 1991). The increased ATP/ADP ratio then closes K^+ channels at the plasma membrane, which results in decreased K^+ efflux and subsequent depolarization of the β-cell (Misler *et al.*, 1986; Cook *et al.*, 1988; Dunne & Petersen, 1991). Depolarization of the β-cell then activates voltage-dependent Ca^{2+} channels, causing an influx of extracellular Ca^{2+} into the β-cell, and an increase in intracellular Ca^{2+} (Prentki & Matschinsky, 1987; Wollheim & Sharp, 1981). Increased intracellular Ca^{2+} activates protein kinases such as the Ca^{2+}-and-calmodulin-dependent protein kinase resulting in insulin exocytosis (Colca *et al.*, 1985; Turk *et al.*, 1987; Easom *et al.*, 1990; Wollheim & Regazzi, 1990).

The muscarinic agonist carbachol binds to the muscarinic receptor to stimulate insulin secretion (Henquin & Nenquin, 1988; Verspohl *et al.*, 1990). Occupancy of the muscarinic receptor activates the coupled phospholipase C at the plasma membrane (Dunlop & Larkins, 1986). Phospholipase C hydrolyses phosphatidylinositol 4,5-bisphosphate with intracellular release of two second messengers, Ins(1,4,5)P_3 and 1,2-diacyl-*sn*-glycerol (DAG) (Turk *et al.*, 1986*b*; Biden *et al.*, 1987; Peter-Riesch *et al.*, 1988; Wolf *et al.*, 1988*b*, 1989); Ins(1,4,5)P_3 stimulates the release of Ca^{2+} from the endoplasmic reticulum of the β-cell (Prentki *et al.*, 1984; Joseph *et al.*, 1984; Wolf *et al.*, 1985, 1986, 1988*a*), whereas diacylglycerol is an endogenous activator of protein kinase C (Ganesan *et al.*, 1990; Easom *et al.*, 1990).

Arachidonic acid metabolites are known to influence insulin secretion (Robertson, 1986; Turk *et al.*, 1987; Metz, 1988*a*). Arachidonic acid is metabolized by the 12-lipoxygenase and the cyclo-oxygenase pathways in islets (Turk *et al.*, 1984*a*,*b*, 1985*b*; Metz, 1985; Robertson, 1988). 12-Hydroxyeicosatetraenoic acid is the major lipoxygenase metabolite, and is believed to have a role in glucose-induced insulin secretion, since specific inhibition of the 12-lipoxygenase inhibits insulin secretion and glucose stimulates its accumulation in islets (Turk *et al.*, 1985*a*; Metz, 1985). Conversely, the cyclo-oxygenase product prostaglandin E_2 inhibits insulin secretion under certain conditions (Robertson, 1986; Laychock, 1990). Unesterified arachidonic acid, the precursor of 12-hydroxyeicosatetraenoic acid and prostaglandin E_2, mobilizes intracellular Ca^{2+} from the endoplasmic reticulum of the β-cell and under certain conditions stimulates insulin release (Wolf *et al.*, 1986, 1987; Metz *et al.*, 1987). Very recently, arachidonic acid has been implicated in the regulation of voltage-dependent Ca^{2+} channels of the β-cell: arachidonic acid, at concentrations present in islet, facilitates voltage-dependent Ca^{2+} entry in the β-cell (Wolf *et al.*, 1991). These observations make arachidonic acid an attractive candidate as part of a signal-transduction mechanism for insulin secretion.

Glucose stimulation increases intracellular unesterified arachidonate concentrations in islets (Wolf *et al.*, 1986, 1987). Two major cellular pathways may account for agonist-induced unesterified arachidonate accumulation. (1) Activated phospholipase A_2 hydrolyses arachidonate from the *sn*-2 position of phospholipids, resulting in accumulation of unesterified

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arachidonate and lysophospholipid. (2) Phospholipase C activation generates diacylglycerol, which can be hydrolysed by diacylglycerol lipase to generate arachidonate. The contribution of either pathway to glucose-induced arachidonate accumulation in islets is unknown. There is indirect evidence that phospholipase A_2 activity is indeed present in islets, but it is not known whether glucose and other secretagogues such as carbachol stimulate phospholipase A_2 in islets (Laychock, 1982; Schrey & Montague, 1983; Dunlop & Larkins, 1984; Metz, 1987*a*).

We now show, for the first time, that carbachol stimulates the accumulation of unesterified arachidonate in pancreatic islets and, if coupled with glucose stimulation, causes an additive increase in non-esterified arachidonate. Furthermore, we show that agonist-induced increases in unesterified arachidonate have a significant Ca^{2+}-independent component. Finally, we demonstrate that the carbachol-induced increase in arachidonate is due to activation of phospholipase A_2 as measured by increased levels of endogenous lysophosphatidylcholine.

EXPERIMENTAL

Materials

Male virus-free Sprague–Dawley rats (200–250 g) were purchased from Charles River (Wilmington, MA, U.S.A.). Collagenase P (lots 20 and 24) was obtained from Boehringer-Mannheim Corp. (Indianapolis, IN, U.S.A.). Tissue-culture medium (CMRL-1066) and 1 M-Hepes were from Gibco Co. (Grand Island, NY, U.S.A.). Newborn-bovine serum was from Hazleton Biologics (Lenexa, KS, U.S.A.). The following compounds were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.): D-glucose, L-glucose, carbachol, Hanks balanced salt solution, penicillin, streptomycin, glutamine, Ficoll, BSA (radioimmunoassay grade, fraction V). RG80267 [1,6-bis(cyclohexyloximinocarbonylamino)hexane] was kindly given by Rhône–Poulenc Rorer Central Research (Horsham, PA, U.S.A.). Phospholipid standards were obtained from Serdary Research Laboratories (Port Huron, MI, U.S.A.). Neutral lipids and fatty acids were purchased from Nu Chek Prep (Elysian, MN, U.S.A.). Organic solvents (h.p.l.c. grade or Optima grade) were provided by Fisher Scientific Co. (Pittsburg, PA, U.S.A.). Other chemicals (except as indicated) were purchased from Sigma or Fisher. The following radioactive compounds were obtained from American Radiolabeled Co. (St Louis, MO, U.S.A.): [5,6,8,9,11,12,14,15-^{3}H(n)]arachidonic acid (180–240 Ci/mmol) and [9,10-^{3}H(n)]palmitic acid (20–60 Ci/mmol). The liquid-scintillation cocktail Universol was purchased from ICN Biomedicals (Costa Mesa, CA, U.S.A.).

Methods

Isolation of islets. In a typical experiment, islets were isolated aseptically from 8–10 male Sprague–Dawley rats. In brief, the bile duct was cannulated and the pancreas was inflated with approx. 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 stereo-microscope. 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 [dialysed and freeze-dried; four layers, of 27 %, 23 %, 20.5 % and 11 %, in Hanks Hepes (25 mM) buffer]. Most 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 of penicillin/ml and 50 μg of streptomycin/ml, 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).

Labelling of islets with [^{3}H]fatty acid. Freshly isolated islets (approx. 3000) were incubated for 24 h under sterile conditions at 37 °C under an atmosphere of air/CO_2 (19:1) in a sterile Petri dish containing 2.5 ml of complete CMRL-1066 and 10 μCi of [^{3}H]arachidonic acid or 50 μCi of [^{3}H]palmitic acid. After labelling, islets were washed five times in fresh modified Krebs–Hepes buffer (25 mM-Hepes, pH 7.4, 115 mM-NaCl, 24 mM-$NaHCO_3$, 5 mM-KCl, 2.5 mM-$CaCl_2$, 1 mM-$MgCl_2$, 0.1 % BSA) supplemented with 3 mM-D-glucose and used immediately as described below.

Perifusion of labelled islets. [^{3}H]Arachidonic acid-labelled islets (200 per condition) were placed on to a mixed cellulose acetate and nitrate filter (type SM, 5 μm pore size; Millipore Corp., Bedford, MA, U.S.A.) in a Swinnex 13 mm perifusion chamber (Millipore) and allowed to equilibrate at a flow rate of 1 ml/min for 30 min at 37 °C in Krebs–Hepes medium supplemented with 3 mM-glucose which was continually gassed with O_2/CO_2 (19:1). The medium was then changed to the experimental condition, and perifusion was continued for another 30 min at a flow rate of 1 ml/min. The perifusate was collected every 2 min in 13 mm × 100 mm borosilicate tubes on ice: its insulin content was assayed by radioimmunoassay, and its radioactivity content was determined 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 determined. The dead-space in the perifusion system was 2.25 ml; data were corrected accordingly. Results are presented as the fractional efflux of radiolabelled arachidonic acid, expressed as the percentage release of total radioactivity incorporated in 200 islets per 2 min fraction (Lacy *et al.*, 1972; Wolf *et al.*, 1989).

Perifusion of [^{3}H]arachidonic acid-labelled islets in the absence of extracellular Ca^{2+}. [^{3}H]Arachidonic acid-labelled islets (200 per condition) were placed on a filter in a perifusion chamber essentially as described above. Islets were then equilibrated for 15 min at 37 °C at a flow rate of 1 ml/min in Krebs–Hepes medium (2.5 mM-$CaCl_2$) supplemented with 3 mM-glucose with continuous gassing with O_2/CO_2 (19:1). This was followed by a 15 min preincubation period with modified Krebs–Hepes medium containing 0.1 mM-EGTA and no Ca^{2+} in order to equilibrate the islets in a medium containing nanomolar concentrations of extracellular Ca^{2+}. Incubation was then continued for another 30 min with the same medium, and the perifusate was collected and processed as above. In experiments where verapamil was used to block Ca^{2+} channels, the 'no Ca^{2+}' solutions were replaced with the appropriate solutions with 30 μM- or 100 μM-verapamil in 1 % ethanol.

Incubation of labelled islets. [^{3}H]Fatty acid-labelled islets (125–200 per condition) were placed in silanized 13 mm × 100 mm round-bottom borosilicate tubes with Teflon screw-caps. Islets 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 O_2/CO_2 (19:1). 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. In some experiments, inhibitors were included in a final dimethyl sulphoxide concentration of 0.2 %, which was also included in the appropriate

controls. Incubation was then continued for 1–30 min. The reaction was stopped by the addition of 2 ml of ice-cold chloroform/methanol (1:2, v/v) supplemented with 0.25% of the anti-oxidant butylated hydroxytoluene. The tubes were then chilled for 15 min in a solid-CO_2/ethanol bath, and stored at −20 °C before analysis.

Extraction of fatty acids and phospholipids. Before extraction, carrier amounts (5 μg) of phospholipids, neutral lipids and fatty acids were added to each tube to aid in recovery, followed by 1 ml of chloroform. Tubes were vortex-mixed (1 min), sonicated (30 min, 4 °C) and vortex-mixed (1 min). Tubes were centrifuged in a refrigerated table-top centrifuge (15 min, 4 °C, 800 *g*). The lower phase was then transferred with a silanized Pasteur pipette to a clean silanized 13 mm × 100 mm conical borosilicate tube. The remaining aqueous upper phase was re-extracted with chloroform (2 × 1 ml) and the extracts were combined with the previous organic phase. The organic phase was then washed with water (1 ml), concentrated twice in a Savant concentrator and reconstituted in 25 μl of chloroform.

One-dimensional t.l.c. analysis of fatty acids and diacylglycerol. Samples were spotted on to the pre-adsorbent zone of channeled silica-gel G t.l.c. plates (20 cm × 20 cm; Analtech, Newark, DE, U.S.A.) which had been activated for 30 min at 110 °C. Plates were developed for 30–45 min in light petroleum (b.p. 30–60 °C)/diethyl ether/acetic acid (70:30:1, by vol.) (Wolf *et al.*, 1986). The radioactivity of the chromatogram was quantified with a Berthold Linear Analyzer 284 (Nashua, NH, U.S.A.) equipped with a position-sensitive proportional high-resolution counter tube (200 mm long, 1380 V) continuously flushed (0.5 l/min) with P10 gas (argon/methane, 9:1) and a 10 mm entrance window. Each t.l.c. lane was scanned simultaneously in its entirety (20 cm) for 30 min. The instrument detected peaks as small as 50–100 d.p.m. with a resolution of 0.5 mm. Data analysis was performed with version 7.19 of the one-dimensional t.l.c. software (Berthold). Radioactive peaks corresponding to non-esterified fatty acids and diacylglycerol were integrated by peak fitting (which automatically calculated and subtracted the background from adjacent regions) and values are expressed as percentages of the counts incorporated into the phospholipid peak. Peak identity was assigned by comparison with iodine-stained non-labelled standards and radiolabelled commercial [^{3}H]arachidonic acid. Typically, the following R_F values were obtained: phospholipids (0), monoacylglycerol (0.19), diacylglycerol (0.45), arachidonic acid (0.63), triacylglycerol (0.88).

H.p.l.c. analysis of eicosanoids. In some experiments, the identity of the [^{3}H]arachidonic acid peak observed on t.l.c. was confirmed by h.p.l.c. (Turk *et al.*, 1984*a*,*b*). The [^{3}H]arachidonic acid peak was scraped from the plate (after detection by the linear analyser), extracted in methanol (2 × 1 ml), and purified by h.p.l.c. on a Varian system (9095 injector, 9010 pump, 9050 detector) equipped with a Whatman C_{18} Partisphere guard column and a C_{18} 5 μm Partisphere 12.5 cm × 4.6 mm cartridge. The following gradient was used, at a flow rate of 1 ml/min: 0–20 min acetonitrile/water/acetic acid (300:700:1, by vol.), 25–45 min acetonitrile/water/acetic acid (530:470:1, by vol.), 50–70 min acetonitrile/acetic acid (1000:1, v/v). Fractions of volume 1 ml were collected and analysed by liquid-scintillation spectrometry after addition of 3 ml of Universol scintillation cocktail (ICN).

Two-dimensional t.l.c. analysis of lysophospholipids. Samples purified from islet labelled with [^{3}H]palmitic acid were applied to 10 cm × 10 cm high-performance HP-K silica-gel t.l.c. plates (Whatman Biosystems Inc., Clifton, NJ, U.S.A.) which had been activated for 30 min at 110 °C. Plates were developed in the first dimension with chloroform/methanol/28% NH_4OH (130:70:11, by vol.) 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, by vol.) for 30 min (Mitchell *et al.*, 1986; Thomas & Holub, 1991). Radioactivity of each plate was quantified 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 with version 4.07 of the two-dimensional t.l.c. software (Berthold). Peaks were integrated after subtracting the background reading of a corresponding and adjacent region. In all cases, radioactivity in the peaks of interest was at least 5 times background. Radioactivity in each peak is expressed as a percentage of the total counts on the plate. The identity of the peaks was assigned by comparison with unlabelled phospholipid standards, commercial radioactive standards, and labelling of the islet phospholipids with the appropriate radiolabelled head group. The following peaks were identified and localized: phosphatidylcholine [R_{F1} (in the first dimension) 0.19, R_{F2} (in the second dimension) 0.38], lysophosphatidylcholine (R_{F1} 0.03, R_{F2} 0.11), phosphatidylethanolamine (R_{F1} 0.31, R_{F2} 0.63), sphingomyelin (R_{F1} 0.09, R_{F2} 0.23), phosphatidylserine (R_{F1} 0.06, R_{F2} 0.39), phosphatidylinositol (R_{F1} 0.01, R_{F2} 0.28), non-esterified fatty acid (R_{F1} 0.28, R_{F2} 0.85), phosphatidic acid (R_{F1} 0.08, R_{F2} 0.63).

Phospholipase A_2 measurement. Phospholipase A_2 activity was measured in islet homogenates. Isolated islets were incubated for 30 min with or without 35 μM-RG80267, rinsed five times with 106 mM-Tes, pH 7.40, and homogenized in 106 mM-Tes (pH 7.40)/50% glycerol. The homogenate was used immediately as the source of islet phospholipase A_2 activity. Phospholipase A_2 activity was measured as the hydrolysis (120 min, 37 °C) of 1-stearoyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonyl-*sn*-phosphatidylcholine (final sp. radioactivity 500 Ci/mol; 2 μM) in 100 mM-Tes (pH 7.40)/2 mM-$CaCl_2$/2 mM-$MgCl_2$/50% glycerol (Hazen *et al.*, 1991). [^{3}H]Arachidonic acid formed was separated from the phospholipid substrate by one-dimensional t.l.c. as described above. Under these conditions, only phospholipase A_2 activity was detected in islet homogenates.

Data analysis. Results are expressed as means ± S.E.M. Statistical analysis was performed with version 4.2 or 5.0 of Statgraphics (STSC Inc., Rockville, MD, U.S.A.). Data were analysed by one-way, two-way or three-way analysis of variance or analysis of covariance 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

Carbachol activation of phospholipase A_2 in islets

Phospholipase A_2 activity was measured by labelling islets for 24 h with [^{3}H]arachidonic acid. Labelled islets were then placed in a perifusion chamber and dynamically challenged with the muscarinic agonist carbachol. Under these conditions, carbachol (0.5 mM) caused a 3-fold peak increase in insulin release, from 0.23 ± 0.01 to 0.74 ± 0.03 μ-unit/min per islet at 2 min ($P < 0.05$), followed by a more sustained 2.5-fold increase ($P < 0.05$) in insulin secretion (Fig. 1*a*). The dynamic release of [^{3}H]arachidonic acid into the perifusate was also assessed in parallel (Fig. 1*b*). Carbachol (0.5 mM) rapidly stimulated [^{3}H]arachidonic acid release: half-maximum release was achieved at 5 min, and maximum release after 14 min of incubation [0.30 ± 0.01% of arachidonate released ($P < 0.05$) versus control value of 0.13 ± 0.01%].

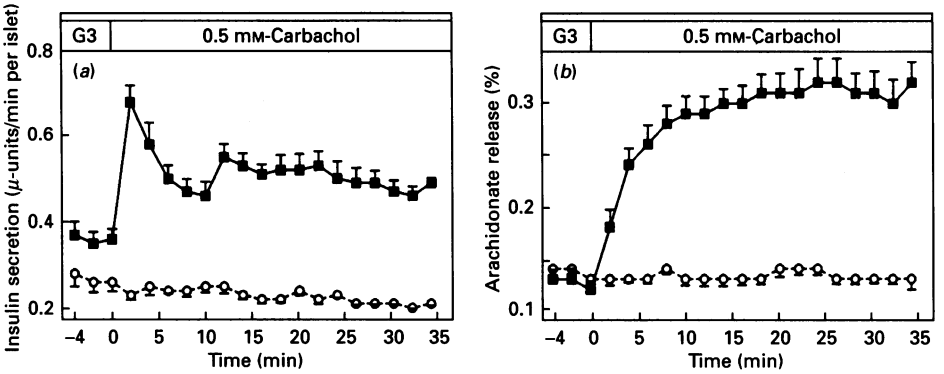

Fig. 1. Effect of carbachol on (*a*) insulin release and (*b*) [^{3}H]arachidonic acid release from perifused islets prelabelled with [^{3}H]arachidonic acid

Isolated islets were prelabelled for 24 h with [^{3}H]arachidonic acid (4 μCi/ml) at 37 °C under air/CO_2 (19:1), placed in a perifusion chamber (200 islets/chamber) and allowed to equilibrate for 30 min at 37 °C in 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-$MgCl_2$, 0.1% BSA) supplemented with 3 mM-glucose (G3) and continuously gassed with O_2/CO_2 (19:1). Islets were then perifused at a flow rate of 1 ml/min for another 30 min with Krebs–Hepes medium supplemented with either 3 mM-glucose (○) or 0.5 mM-carbachol and 3 mM-glucose (■). Perifusate was collected every 2 min and assayed for its insulin and [^{3}H]arachidonic acid contents. Results are shown as means ± S.E.M. from 4 experiments, and are expressed as μ-units of insulin/min per islet (*a*) or fractional release of [^{3}H]arachidonate (% of total incorporated; *b*). Total [^{3}H]arachidonic acid incorporation into 200 islets was 273 049 ± 26078 c.p.m. ($n = 8$).

[^{3}H]Arachidonic acid accumulation in islets was measured in a static incubation design: in this method, unesterified [^{3}H]arachidonic acid is extracted, separated from phospholipids and neutral lipids by t.l.c. and quantified by using a linear scanner, thus providing an index of intracellular accumulation of [^{3}H]arachidonic acid. Quantification of the radioactivity present in each peak on the t.l.c. plate with the linear scanner was very sensitive and precise: as little as 50–100 d.p.m. could be easily detected with a resolution of 0.5 mm. Furthermore, the radioactive peaks of [^{3}H]arachidonic acid were clearly above background (Fig. 2). H.p.l.c. analysis of the [^{3}H]arachidonic acid peak on the t.l.c. plate demonstrated that most of the radioactivity was arachidonic acid and not lipoxygenase or cyclo-oxygenase metabolites (Fig. 3) As shown in Fig. 4, carbachol as well as glucose caused an increase in [^{3}H]arachidonic acid accumulation compared with the 3 mM-glucose control: at 15 min of incubation, glucose (28 mM) increased [^{3}H]arachidonic acid accumulation to 160.1 ± 22.6% of the 3 mM-glucose control, and carbachol (0.5 mM) increased it to 295.5 ± 38.2% of control. The combination of glucose (28 mM) and carbachol (0.5 mM) tended to cause a greater increase in [^{3}H]arachidonic acid accumulation than with either secretagogue alone (263 ± 11.4%, 188.2 ± 12.0% and 294.1 ± 22.2% of the 3 mM-glucose control at 2, 5 and 15 min respectively). L-Glucose (25 mM) had no effect on [^{3}H]arachidonic acid accumulation (results not shown).

Glucose (28 mM), carbachol (0.5 mM) and glucose (28 mM) + carbachol (0.5 mM) significantly increased insulin secretion (Fig. 5*a*): secretagogue-induced insulin secretion over basal (3 mM-glucose) was 5-fold for glucose, 3-fold for carbachol and 12-fold for the combination of glucose + carbachol ($P < 0.05$ versus 3 mM-glucose, under all conditions). [^{3}H]Arachidonic acid release was also significantly increased by these secretagogues (Fig. 5*b*). Glucose (28 mM) caused a 1.7-fold increase in [^{3}H]arachidonic acid release over a 30 min incubation period compared with 3 mM-glucose ($P < 0.05$). Carbachol (0.5 mM) resulted in a 2.6-fold increase in [^{3}H]arachidonic acid release compared with the 3 mM-glucose baseline ($P < 0.05$). Finally, the combination of glucose (28 mM) + carbachol (0.5 mM) resulted in a 3.5-fold increase in [^{3}H]arachidonic acid release compared with 3 mM-glucose ($P < 0.05$).

Ca^{2+}-dependency of carbachol activation of phospholipase A_2

Extracellular Ca^{2+} is required for insulin secretion (Wollheim & Sharp, 1981). In general, most phospholipases A_2 are Ca^{2+}-dependent (Clark *et al.*, 1991). The Ca^{2+}-dependency of secretagogue-induced [^{3}H]arachidonic acid release was examined in a perifusion model which allows the simultaneous determination of insulin release as well as of arachidonic acid release.

The omission of extracellular Ca^{2+} combined with the addition of the Ca^{2+} chelator EGTA (0.1 mM) abolished insulin secretion induced by 28 mM-glucose (0.29 ± 0.04 μ-unit of insulin/min per islet), 0.5 mM-carbachol (0.21 ± 0.02 μ-unit/min per islet) and 28 mM-glucose + 0.5 mM-carbachol (0.24 ± 0.03 μ-unit/min per islet) but had no effect on basal insulin release (0.28 ± 0.03 μ-unit/min per islet). In the absence of extracellular Ca^{2+}, there was significant secretagogue-induced release of [^{3}H]arachidonic acid compared with that with 3 mM-glucose for each secretagogue alone as well as with the combination. Glucose (28 mM) significantly increased [^{3}H]arachidonic acid release (0.18 ± 0.005 versus 0.15 ± 0.002% at 3 mM-glucose; $P < 0.05$). Carbachol also increased [^{3}H]arachidonic acid release (0.17 ± 0.003%; $P < 0.05$ versus 3 mM-glucose control). The combination of glucose and carbachol increased [^{3}H]arachidonic acid release by 53% compared with control (0.23 ± 0.01%; $P < 0.05$ versus 3 mM-glucose control).

Ca^{2+}-dependency was also investigated by using the Ca^{2+}-channel blocker verapamil. Verapamil at concentrations of 30 μM (results not shown) and 100 μM, which block secretagogue-induced insulin secretion, only inhibited by 48% the glucose (28 mM) + carbachol (0.5 mM)-induced [^{3}H]arachidonic acid release ($P < 0.05$; Fig. 6). These observations indicate that secretagogue-induced [^{3}H]arachidonic acid release has a large component that is independent of extracellular Ca^{2+}.

Carbachol stimulation of lysophosphatidylcholine accumulation in islets

Agonist-induced stimulation of phospholipase A_2 results in the intracellular accumulation of unesterified arachidonate and of lysophospholipid. Demonstration of intracellular lysophospholipid accumulation has generally been difficult to document, due in part to methodological considerations as well as to rapid reacylation. We have used [^{3}H]palmitic acid labelling of islets to measure lysophospholipid accumulation in islets in response to secretagogues. Under these conditions, [^{3}H]palmitic acid, a saturated fatty acid, is preferentially incorporated in the *sn*-1 position of the glycerol backbone of the phospholipid. By

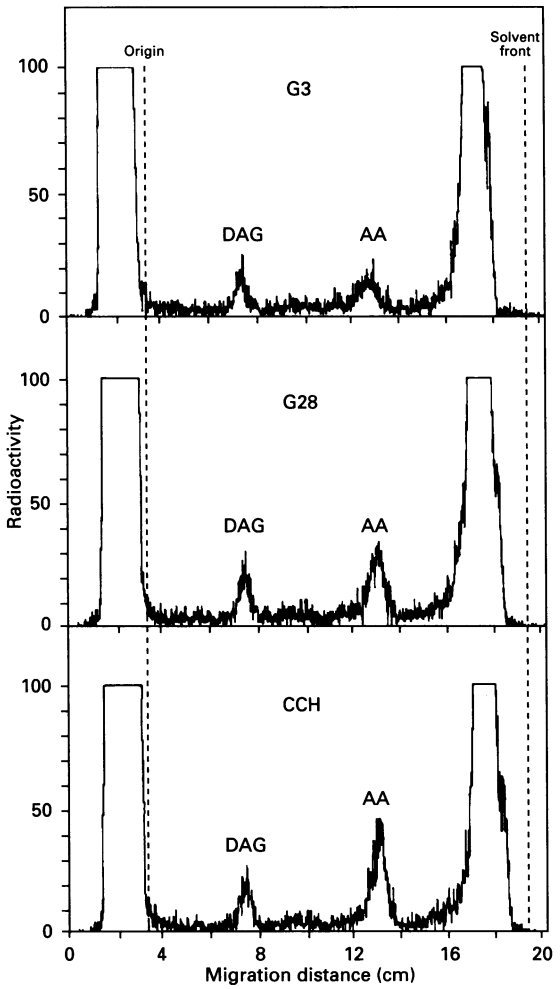

Fig. 2. Linear-analyser quantification of t.l.c. separation of [^{3}H]arachidonic acid from islets prelabelled with [^{3}H]arachidonic acid

Islets were labelled with [^{3}H]arachidonic acid as in Fig. 1. Islets (125) were placed in silanized tubes, preincubated for 30 min in Krebs–Hepes medium supplemented with 3 mM-glucose, and then incubated 30 min in fresh Krebs–Hepes medium supplemented with 3 mM-glucose (top panel: G3), 28 mM-glucose (middle panel: G28) or 0.5 mM-carbachol + 3 mM-glucose (lower panel: CCH). Neutral lipids and unesterified fatty acids were extracted as described in the Experimental section and analysed by one-dimensional t.l.c. with a mobile phase of light petroleum/diethyl ether/acetic acid (70:30:1, by vol.). Radioactivity on the t.l.c. plates was quantified with a Berthold linear analyser and is expressed as counts of radioactivity as a function of the migration distance on the t.l.c. plate from a typical experiment (total radioactivity was similar in all three conditions). The peak at the origin is the non-migrating phospholipid peak, and the peak close to the solvent front is triacylglycerols. Abbreviations: DAG, diacylglycerol; AA, unesterified arachidonic acid.

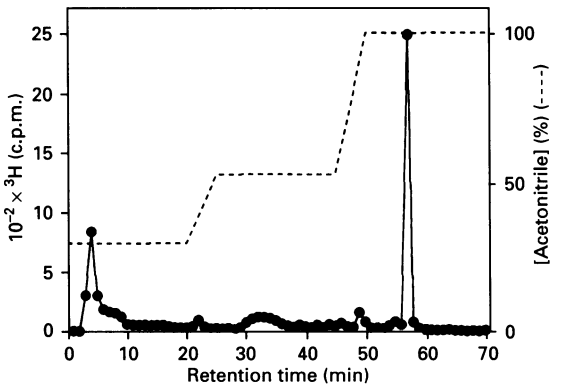

Fig. 3. Reverse-phase h.p.l.c. analysis of ^{3}H-labelled eicosanoids from islets prelabelled with [^{3}H]arachidonic acid and stimulated with 28 mM-glucose and 0.5 mM-carbachol

^{3}H-labelled material with the mobility of arachidonic acid on t.l.c. (see Fig. 2) was extracted, and analysed by reverse-phase h.p.l.c. as described under Methods. Cyclo-oxygenase products are eluted in the first solvent step (0–20 min), lipoxygenase products in the second solvent step (25–45 min), and arachidonic acid is eluted in the last solvent step (50–70 min). Results are representative of three experiments.

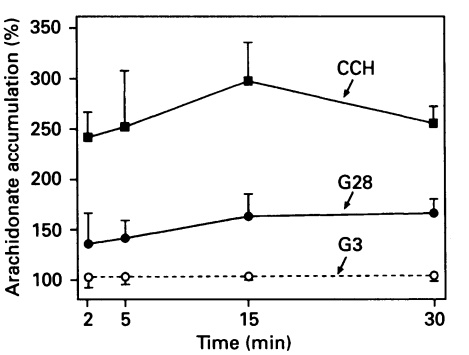

Fig. 4. Effect of carbachol and glucose on [^{3}H]arachidonic acid accumulation in islets prelabelled with [^{3}H]arachidonic acid

Islets were labelled with [^{3}H]arachidonic acid, incubated, and unesterified [^{3}H]arachidonic acid was extracted and analysed as in Fig. 2. Results are shown as means ± S.E.M. of arachidonate accumulation (% of 3 mM-glucose control) for islets incubated with 3 mM-glucose (○; G3), 28 mM-glucose (●; G28), or 0.5 mM-carbachol + 3 mM-glucose (■; CCH) for 5–10 observations per time point.

use of two-dimensional t.l.c. to separate lysophospholipids and phospholipids, combined with sensitive and high-resolution two-dimensional radioactive linear scanning, carbachol (0.5 mM) was found to cause a rapid increase in lysophosphatidylcholine compared with 3 mM-glucose (Table 1). No concomitant decrease in phosphatidylcholine or increase in other lysophospholipids was observed (results not shown). At 1 and 2 min, 28 mM-glucose alone had no effect on lysophosphatidylcholine accumulation (0.48 ± 0.06 and 0.42 ± 0.07% of total phospholipids at 1 and 2 min) compared with 3 mM-glucose (0.51 ± 0.12 and 0.40 ± 0.06% at 1 and 2 min). At 30 min, 28 mM-glucose caused a modest but not significant increase in lysophosphatidylcholine (Table 1).

Diacylglycerol as a source of arachidonic acid in islets

Steady-state levels of [*arachidonoyl*-^{3}H]diacylglycerol did not change after incubation with 28 mM-glucose, 0.5 mM-carbachol and the combination of both secretagogues (see Fig. 2). The possibility that secretagogues were stimulating the metabolism of diacylglycerol to arachidonic acid was investigated. There are two known pathways of diacylglycerol metabolism: phosphorylation to phosphatidic acid by diacylglycerol kinase, and hydrolysis by diacylglycerol lipase, which generates unesterified fatty acid. Mono-olein has been shown to inhibit diacylglycerol kinase (Bishop & Bell, 1986; Wolf *et al.*, 1989), and RG80267 is a widely used inhibitor of diacylglycerol lipase (Capito *et al.*, 1989; Wolf *et al.*, 1989). Mono-olein (100 μM) did not affect glucose (28 mM) + carbachol (0.5 mM)-induced

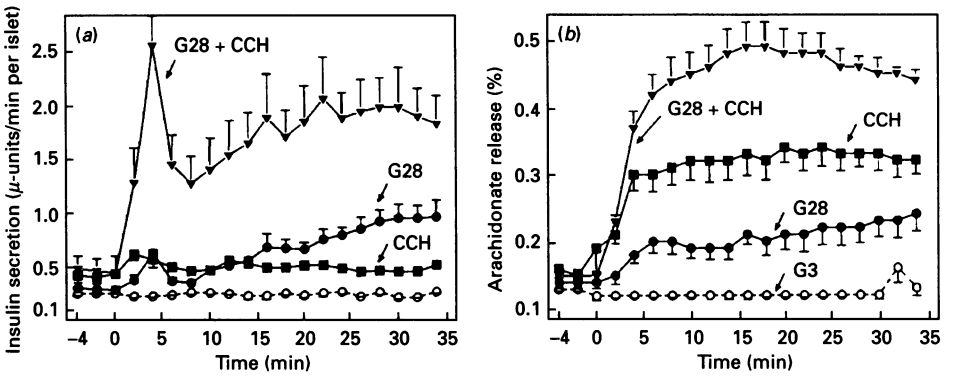

Fig. 5. Effect of carbachol and glucose on (*a*) insulin secretion and (*b*) [^{3}H]arachidonic acid release from perifused islets prelabelled with [^{3}H]arachidonic acid

Islets were labelled with [^{3}H]arachidonic acid and perifused as described in the Experimental section. (*a*) Secretagogue-induced insulin secretion; (*b*) secretagogue-induced [^{3}H]arachidonate release. Secretion results are shown as means ± S.E.M. of insulin secretion (μ-units of insulin/min per islet) for islets incubated with 3 mM-glucose (G3: ○), 28 mM-glucose (G28: ●), 0.5 mM-carbachol + 3 mM-glucose (CCH: ■), or 28 mM-glucose + 0.5 mM-carbachol (G28 + CCH: ▼) from 3 observations per time point. Arachidonate release is shown as the mean (± S.E.M.) fractional arachidonate release (% of total [^{3}H]arachidonate acid incorporated) from islets incubated with 3 mM-glucose (G3: ○), 28 mM-glucose (G28: ●), 0.5 mM-carbachol + 3 mM-glucose (CCH: ■), or 28 mM-glucose + 0.5 mM-carbachol (G28 + CCH: ▼) from 3 observations per time point. Total [^{3}H]arachidonic acid incorporated per 200 islets was 313884 ± 21784 c.p.m. (n = 24).

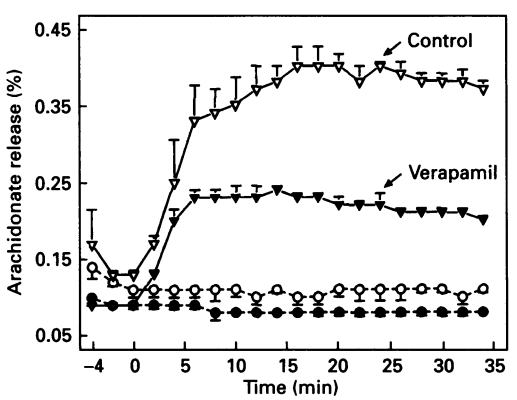

Fig. 6. Effect of verapamil on glucose + carbachol-induced [^{3}H]arachidonic acid release from perifused islets prelabelled with [^{3}H]arachidonic acid

Islets were labelled with [^{3}H]arachidonic acid as described in Fig. 1 and placed in perifusion chambers (200 islets/chamber). Chambers were equilibrated for 15 min with Krebs–Hepes medium supplemented with 3 mM-glucose and then for another 15 min with 3 mM-glucose + 1% ethanol or 3 mM-glucose + 100 μM-verapamil in 1% ethanol. Chambers were then perifused for another 30 min as described in Fig. 1. Results are shown as means ± S.E.M. of fractional arachidonate release (% of total [^{3}H]arachidonic acid incorporated into 200 islets) from islets incubated with 3 mM-glucose + 1% ethanol (G3 control: ○), 3 mM-glucose + 100 μM-verapamil (G3 verapamil: ●), 28 mM-glucose + 0.5 mM-carbachol + 1% ethanol (G28 + CCH control: ▽), or 28 mM-glucose + 0.5 mM-carbachol + 100 μM-verapamil (G28 + CCH verapamil: ▼) from two experiments. Total [^{3}H]arachidonic acid incorporated per 200 islets was 447654 ± 52397 c.p.m. (n = 8).

Table 1. Effect of carbachol and glucose on lysophosphatidylcholine accumulation in islets prelabelled with [^{3}H]palmitic acid

Islets were labelled with [^{3}H]palmitic acid (20 μCi/ml) for 24 h as described in the Experimental section and incubated (200 islets/condition) with carbachol and/or glucose. Phospholipids were extracted and separated by two-dimensional t.l.c. as described under 'Methods'. Radioactivity in each peak was measured by two-dimensional radioactivity scanning with a Berthold Linear Analyzer. Results are expressed as means ± S.E.M. of radioactivity in lysophosphatidylcholine (as % of 3 mM-glucose control). Radioactivity incorporated into lysophosphatidylcholine under 3 mM-glucose control conditions at 1, 2 and 30 min was 0.21%, 0.29% and 0.25% respectively of all radioactivity incorporated into each 200-islets sample (604642 ± 33206 counts; n = 32). *P < 0.05 versus 3 mM-glucose control.

Condition	Time (min)	Lysophosphatidylcholine (% of 3 mM-glucose)	n
3 mM-glucose	1	100.0 ± 17.1	2
28 mM-glucose + 0.5 mM-carbachol	1	139.1 ± 22.0	2
3 mM-glucose	2	100.0 ± 4.5	3
28 mM-glucose + 0.5 mM-carbachol	2	121.2 ± 2.9	3
3 mM-glucose	30	100.0 ± 5.6	7
28 mM-glucose	30	130.5 ± 15.8	4
0.5 mM-carbachol	30	164.9 ± 26.1*	4
28 mM-glucose + 0.5 mM-carbachol	30	154.8 ± 19.5*	7

accumulation of [^{3}H]arachidonic acid (results not shown). RG80267, a purported inhibitor of diacylglycerol lipase, had complex effects on secretagogue-induced [^{3}H]arachidonic acid accumulation (Table 2). RG80267 concentrations greater than 35 μM are toxic to islets (Capito *et al.*, 1989). RG80267 (35 μM) completely abolished glucose-induced [^{3}H]arachidonic acid accumulation, but only partially inhibited carbachol-induced [^{3}H]arachidonic acid accumulation (a doubling was still observed compared with control). The combination of glucose and carbachol in the presence of RG80267, however, was synergistic for [^{3}H]arachidonic acid accumulation (Table 2). These observations indicate that secretagogue-induced accumulation of [^{3}H]arachidonic acid is partially due to diacylglycerol hydrolysis.

The specificity of RG80267 has been questioned, since it affects glucose oxidation in islets (Capito *et al.*, 1989). We have measured its effects on phospholipase A_2 activity in islet homogenates. A nearly 30% inhibition of phospholipase A_2 was observed at 35 μM-RG80267 (803.6 ± 72.2 pmol of phosphatidylcholine hydrolysed/mg of protein in controls versus 547.5 ± 30.8 pmol/mg in treated islets; n = 5).

Table 2. Effect of the diacylglycerol lipase inhibitor RG80267 on secretagogue-induced [^{3}H]arachidonic acid accumulation in islets prelabelled with [^{3}H]arachidonic acid

Islets were labelled with [^{3}H]arachidonic acid, incubated for 30 min with control or 35 μM-RG80267, and unesterified [^{3}H]arachidonic acid was extracted and analysed as in Fig. 2. Results are shown as mean ± S.E.M. of arachidonate accumulation (% of total incorporated into phospholipids) for 3–10 observations per condition: *$P < 0.05$ versus 3 mM-glucose control; †$P < 0.05$ versus 3 mM-glucose + 35 μM-RG80267; ‡$P < 0.05$ versus carbachol control; §$P < 0.05$ versus carbachol + glucose control.

Condition	Arachidonic acid accumulation (% of total incorporated in phospholipids)	
	Control	RG80267 (35 μM)
3 mM-glucose	0.48 ± 0.03	0.33 ± 0.04
28 mM-glucose	0.83 ± 0.03	0.30 ± 0.03
3 mM-glucose + 0.5 mM-carbachol	1.33 ± 0.17*	0.60 ± 0.02‡
0.5 mM-carbachol + 28 mM-glucose	2.49 ± 0.28*	0.99 ± 0.15†§

DISCUSSION

We have shown that carbachol and glucose, at concentrations which stimulate insulin secretion, cause arachidonic acid accumulation in isolated islets of Langerhans. Agonist-induced accumulation of arachidonic acid is rapid, and there is a significant component of arachidonic acid release which is independent of extracellular Ca^{2+}. We also demonstrate that carbachol-induced arachidonic acid accumulation is in part due to phospholipase A_2 activation, as demonstrated by the increased accumulation of lysophosphatidylcholine. Diacylglycerol hydrolysis also contributes to secretagogue-induced accumulation of arachidonic acid.

Phospholipase A_2 activity has been previously identified in rat islet homogenates using [^{14}C]oleate-labelled phospholipids from *Escherichia coli* as substrate: phospholipase A_2 was Ca^{2+}-dependent and appeared to be stimulated by glucose (Laychock, 1982). Earlier studies have shown that glucose stimulation of intact islets results in the rapid and significant accumulation of endogenous arachidonic acid as measured by g.l.c.–mass spectrometry (Wolf *et al.*, 1986, 1987). Our study provides the first demonstration that carbachol causes the accumulation of arachidonic acid and lysophosphatidylcholine in intact islets, and therefore stimulates phospholipase A_2. Lysophosphatidylcholine accumulation was only seen in islets radiolabelled with [^{3}H]palmitic acid and not with other fatty acid labels: this observation is not entirely surprising, since islet lysophosphatidylcholine consists entirely of palmitate and stearate (Turk *et al.*, 1986*a*), and furthermore agonist stimulation of islets causes large accumulation of unesterified stearate, but not palmitate (Wolf *et al.*, 1991).

The amplitude of carbachol-induced accumulation of lysophosphatidylcholine (50–60 % increase) is less than the amplitude of carbachol-induced accumulation of unesterified arachidonic acid. A likely explanation for this discrepancy is that, in addition to phospholipase A_2 activation, diacylglycerol hydrolysis is contributing to arachidonic acid accumulation. Alternatively, it is known that lysophosphatidylcholine is rapidly reacylated (Metz, 1986*b*, 1987*b*) or is further hydrolysed by lysophospholipases (Badiani & Arthur, 1991; Pind & Kuksis, 1991). In most other cellular systems it has proved very difficult to demonstrate agonist-induced increases in lysophospholipids in intact cells (Laychock, 1989). It is also possible that phosphatidic acid (derived from phospholipase D activation) is the source of arachidonic acid (Dunlop & Metz, 1989; Metz & Dunlop, 1990; Konrad *et al.*, 1991).

The possibility that glucose-induced unesterified arachidonate accumulation reflects the sequential action of phospholipase C and diacylglycerol lipase merits consideration (Schrey & Montague, 1983). Phospholipase C activity is clearly present in islets and is stimulated by glucose and carbachol (Turk *et al.*, 1986*b*; Biden *et al.*, 1987; Peter-Riesch *et al.*, 1988; Wolf *et al.*, 1988*b*, 1989). We could not detect any increase in this study in [*arachidonoyl*-^{3}H]diacylglycerol induced by glucose, carbachol, or the combination of the two. These data are consistent with our previous studies using g.l.c.–mass-spectrometric methods as well as enzymic methods, which have failed to detect any glucose-induced increase in the arachidonoyl content of 1,2-diacyl-*sn*-glycerol (Peter-Riesch *et al.*, 1988; Wolf *et al.*, 1989, 1990). Other studies using similar approaches have also failed to detect glucose-induced increases in 1,2-diacyl-*sn*-glycerol in islets and insulin-secreting HIT-T15 cell lines (Peter-Riesch *et al.*, 1988). Nevertheless RG80267, an inhibitor of diacylglycerol lipase, inhibits glucose- and carbachol-induced insulin secretion (Capito *et al.*, 1989; Wolf *et al.*, 1989, 1990), as well as arachidonic acid accumulation (Table 2). A definitive interpretation of these studies is, however, obscured by the fact that RG 80267 interferes with glucose metabolism (Capito *et al.*, 1989; Wolf *et al.*, 1990), and, as shown in the present study, partially inhibits islet phospholipase A_2.

The precise role of phospholipase A_2 in insulin secretion has not been completely elucidated. Certainly, carbachol stimulates phospholipase A_2 activity in islets. There is strong evidence implicating its end-products arachidonic acid and lysophospholipids in insulin secretion. Lysophospholipids have been implicated as mediators of insulin secretion (Metz, 1986*b*). In particular, lysophosphatidylcholine and lysophosphatidylinositol have been shown to induce $^{45}Ca^{2+}$ efflux and insulin secretion from islets (Metz, 1986*a*,*c*; Fujimoto & Metz, 1987; Fujimoto & Teague, 1989). Arachidonic acid has a number of intracellular effects in islets consistent with its proposed role as a second messenger. Arachidonic acid, at concentrations present in islets, clearly mobilizes Ca^{2+} from the endoplasmic reticulum, resulting in an increase in intracellular Ca^{2+}, independently of any interaction with the second messenger Ins(1,4,5)P_3 (Wolf *et al.*, 1986, 1987; Metz *et al.*, 1987). This Ca^{2+}-mobilizing effect of arachidonic acid has now been demonstrated in numerous other cell lines (Chan & Turk, 1987; Wolf *et al.*, 1988*a*). Arachidonic acid may also stimulate protein kinase C in islets, as well as in other systems (Metz, 1988*b*; Buday & Faragó, 1990; Shearman *et al.*, 1991; Shinomura *et al.*, 1991). Finally, we have recently shown that arachidonic acid, at physiological concentrations, amplifies the insulin-secretory response of islets exposed to submaximal depolarizing concentrations of K^+, indicating that arachidonic acid may directly facilitate voltage-dependent Ca^{2+} entry in islets (Wolf *et al.*, 1991).

In conclusion, we have shown that the insulin secretagogue carbachol causes arachidonic acid accumulation. Carbachol activates phospholipase A_2 in islets, resulting in accumulation of unesterified arachidonic acid and lysophosphatidylcholine. Carbachol-induced arachidonic acid accumulation also appears to involve diacylglycerol hydrolysis. These observations suggest that phospholipase A_2 may have an important role in muscarinic-induced insulin secretion.

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REFERENCES

Ashcroft, S. J. (1980) Diabetologia **18**, 5–15
Badiani, K. & Arthur, G. (1991) Biochem. J. **275**, 393–398
Biden, T. J., Peter-Riesch, B., Schlegel, W. & Wollheim, C. B. (1987) J. Biol. Chem. **262**, 3567–3571
Bishop, W. R. & Bell, R. M. (1986) J. Biol. Chem. **261**, 12513–12519
Buday, L. & Faragó, A. (1990) FEBS Lett. **276**, 223–226
Capito, K., Hansen, S. E., Hedeskov, C. J. & Thams, P. (1989) Diabetologia **32**, 111–117
Chan, K. M. & Turk, J. (1987) Biochim. Biophys. Acta **928**, 186–193
Clark, J. D., Lin, L.-L., Kriz, R. W., Ramesha, C. S., Sultzman, L. A., Lin, A. Y., Milona, N. & Knopf, J. L. (1991) Cell **65**, 1043–1051
Colca, J. R., Wolf, B. A., Comens, P. G. & McDaniel, M. L. (1985) Biochem. J. **228**, 529–536
Cook, D. L., Satin, L. S., Ashford, M. L. & Hales, C. N. (1988) Diabetes **37**, 495–498
Dunlop, M. E. & Metz, S. A. (1989) Biochem. Biophys. Res. Commun. **163**, 922–928
Dunlop, M. E. & Larkins, R. G. (1984) Biochem. Biophys. Res. Commun. **120**, 820–827
Dunlop, M. E. & Larkins, R. G. (1986) Biochem. J. **240**, 731–737
Dunne, M. J. & Petersen, O. H. (1991) Biochim. Biophys. Acta **1071**, 67–82
Easom, R. A., Landt, M., Colca, J. R., Hughes, J. H., Turk, J. & McDaniel, M. (1990) J. Biol. Chem. **265**, 14938–14946
Fujimoto, W. Y. & Metz, S. A. (1987) Endocrinology (Baltimore) **120**, 1750–1757
Fujimoto, W. Y. & Teague, J. (1989) Diabetes **38**, 625–628
Ganesan, S., Calle, R., Zawalich, K., Smallwood, J. I., Zawalich, W. S. & Rasmussen, H. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 9893–9897
Garcia, M. C., Hermans, M. P. & Henquin, J. C. (1988) Biochem. J. **254**, 211–218
Ghosh, A., Ronner, P., Cheong, E., Khalid, P. & Matschinsky, F. M. (1991) J. Biol. Chem. **266**, 22887–22892
Hazen, S. L., Loeb, L. A. & Gross, R. W. (1991) Methods Enzymol. **197**, 400–410
Hedeskov, C. J. (1980) Physiol. Rev. **60**, 442–509
Henquin, J. C. & Nenquin, M. (1988) FEBS Lett. **236**, 89–92
Joseph, S. K., Williams, R. J., Corkey, B. E., Matschinsky, F. M. & Williamson, J. R. (1984) J. Biol. Chem. **259**, 12952–12955
Konrad, R. J., Jolly, Y. C. & Wolf, B. A. (1991) Biochem. Biophys. Res. Commun. **180**, 960–966
Lacy, P. E. & Kostianovsky, M. (1967) Diabetes **16**, 35–43
Lacy, P. E., Walker, M. M. & Fink, C. J. (1972) Diabetes **21**, 987–998
Laychock, S. G. (1982) Cell Calcium **3**, 43–54
Laychock, S. G. (1989) Curr. Top. Cell. Regul. **30**, 203–242
Laychock, S. G. (1990) Mol. Pharmacol. **37**, 928–936
MacDonald, M. J. (1990) Diabetes **39**, 1461–1466
Malaisse, W. J., Sener, A., Herchuelz, A. & Hutton, J. C. (1979) Metab. Clin. Exp. **28**, 373–386
Matschinsky, F. M. (1990) Diabetes **39**, 647–652
McDaniel, M. L., Colca, J. R., Kotagal, N. & Lacy, P. E. (1983) Methods Enzymol. **98**, 182–200
Meglasson, M. D. & Matschinsky, F. M. (1986) Diabetes Metab. Rev. **2**, 163–214
Metz, S. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 198–202
Metz, S. A. (1986*a*) Diabetes **35**, 808–817
Metz, S. A. (1986*b*) J. Pharmacol. Exp. Ther. **238**, 809–818
Metz, S. A. (1986*c*) J. Pharmacol. Exp. Ther. **238**, 819–832
Metz, S. A. (1987*a*) Adv. Prostaglandin Thromboxane Leukotriene Res. **17B**, 668–676
Metz, S. A. (1987*b*) Biochem. J. **241**, 863–869
Metz, S. A. (1988*a*) Prostaglandins Leukotrienes Essent. Fatty Acids **32**, 187–202
Metz, S. A. (1988*b*) Diabetes **37**, 1453–1469
Metz, S. A. & Dunlop, M. (1990) Biochem. J. **270**, 427–435
Metz, S. A., Draznin, B., Sussman, K. E. & Leitner, J. W. (1987) Biochem. Biophys. Res. Commun. **142**, 251–258
Misler, S., Falke, L. C., Gillis, K. & McDaniel, M. L. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7119–7123
Mitchell, K. T., Ferrell, J. E. & Huestis, W. H. (1986) Anal. Biochem. **158**, 447–453
Peter-Riesch, B., Fathi, M., Schlegel, W. & Wollheim, C. B. (1988) J. Clin. Invest. **81**, 1154–1161
Pind, S. & Kuksis, A. (1991) Biochem. Cell Biol. **69**, 346–357
Prentki, M. & Matschinsky, F. M. (1987) Physiol. Rev. **67**, 1185–1248
Prentki, M., Biden, T. J., Janjic, D., Irvine, R. F., Berridge, M. J. & Wollheim, C. B. (1984) Nature (London) **309**, 562–564
Rajan, A. S., Aguilar-Bryan, L., Nelson, D. A., Yaney, G. C., Hsu, W. H., Kunze, D. L. & Boyd, A. E. (1990) Diabetes Care **13**, 340–363
Robertson, R. P. (1986) Diabetes Metab. Rev. **2**, 261–296
Robertson, R. P. (1988) Diabetes **37**, 367–370
Schrey, M. P. & Montague, W. (1983) Biochem. J. **216**, 433–441
Shearman, M. S., Shinomura, T., Oda, T. & Nishizuka, Y. (1991) FEBS Lett. **279**, 261–264
Shinomura, T., Asaoka, Y., Oka, M., Yoshida, K. & Nishizuka, Y. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 5149–5153
Thomas, L. M. & Holub, B. J. (1991) Biochim. Biophys. Acta. **1081**, 92–98
Turk, J., Colca, J. R., Kotagal, N. & McDaniel, M. L. (1984*a*) Biochim. Biophys. Acta **794**, 110–124
Turk, J., Colca, J. R., Kotagal, N. & McDaniel, M. L. (1984*b*) Biochim. Biophys. Acta **794**, 125–136
Turk, J., Colca, J. R. & McDaniel, M. L. (1985*a*) Biochim. Biophys. Acta **834**, 23–36
Turk, J., Wolf, B. A., Comens, P. G., Colca, J., Jakschik, B. & McDaniel, M. L. (1985*b*) Biochim. Biophys. Acta **835**, 1–17
Turk, J., Wolf, B. A., Lefkowith, J. B., Stump, W. T. & McDaniel, M. L. (1986*a*) Biochim. Biophys. Acta **879**, 399–409
Turk, J., Wolf, B. A. & McDaniel, M. L. (1986*b*) Biochem. J. **237**, 259–263
Turk, J., Wolf, B. A. & McDaniel, M. L. (1987) Prog. Lipid Res. **26**, 125–181
Verspohl, E. J., Tacke, R., Mutschler, E. & Lambrecht, G. (1990) Eur. J. Pharmacol. **178**, 303–311
Wolf, B. A., Comens, P. G., Ackermann, K. E., Sherman, W. R. & McDaniel, M. L. (1985) Biochem. J. **227**, 965–969
Wolf, B. A., Turk, J., Sherman, W. R. & McDaniel, M. L. (1986) J. Biol. Chem. **261**, 3501–3511
Wolf, B. A., Turk, J., Comens, P. G., Sherman, W. R. & McDaniel, M. L. (1987) Ann. N.Y. Acad. Sci. **494**, 168–170
Wolf, B. A., Colca, J. R., Turk, J., Florholmen, J. & McDaniel, M. L. (1988*a*) Am. J. Physiol. **254**, E121–E136
Wolf, B. A., Florholmen, J., Turk, J. & McDaniel, M. L. (1988*b*) J. Biol. Chem. **263**, 3565–3575
Wolf, B. A., Easom, R. A., Hughes, J. H., McDaniel, M. L. & Turk, J. (1989) Biochemistry **28**, 4291–4301
Wolf, B. A., Easom, R. A., McDaniel, M. L. & Turk, J. (1990) J. Clin. Invest. **85**, 482–490
Wolf, B. A., Pasquale, S. M. & Turk, J. (1991) Biochemistry **30**, 6372–6379
Wollheim, C. B. & Regazzi, R. (1990) FEBS Lett. **268**, 376–380
Wollheim, C. B. & Sharp, G. W. (1981) Physiol. Rev. **61**, 914–973
Zawalich, W. S. & Rasmussen, H. (1990) Mol. Cell. Endocrinol. **70**, 119–137

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