Ca^{2+}-Independent Phospholipase A_2 Contributes to the Insulinotropic Action of Cholecystokinin-8 in Rat Islets

Dissociation From the Mechanism of Carbachol

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Insulin secretion induced by cholecystokinin-8 (CCK-8) was recently suggested to involve phospholipase A_2 (PLA_2) activation. In this study, we examined whether CCK-8 stimulates the Ca^{2+}-independent form of PLA_2 in isolated rat islets, in comparison with stimulation by the PLA_2-activating cholinergic agonist carbachol. We found that CCK-8 (100 nmol/l; 5.6 mmol/l glucose) induces lysophosphatidylcholine accumulation from [^{3}H]palmitate-prelabeled islets (170 ± 39%; $P = 0.003$) as well as arachidonic acid (AA) efflux from [^{3}H]AA-prelabeled islets (190 ± 13%; $P < 0.001$), and that *p*-amylcinnamoylantranilic acid (ACA) (50 µmol/l)–mediated PLA_2 inhibition reduces CCK-8–induced AA efflux (52 ± 11%; $P = 0.001$) and insulin secretion (67 ± 16%; $P < 0.001$). Neither the Ca^{2+} channel antagonist verapamil (100 µmol/l) nor the Ca^{2+}ATPase inhibitor thapsigargin (1 µmol/l) affected CCK-8–induced AA efflux and insulin secretion. Furthermore, despite removal of extracellular Ca^{2+}, CCK-8 still increased AA efflux (48 ± 14%; $P = 0.006$) and insulin secretion (105 ± 46%; $P = 0.025$). In contrast, carbachol (100 µmol/l)-stimulated AA efflux was reduced by verapamil by 36 ± 6% ($P < 0.001$) and abolished by removal of extracellular Ca^{2+}. Overnight protein kinase C (PKC) downregulation by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (500 nmol/l) reduced CCK-8–induced AA efflux (45 ± 12%; $P = 0.003$) and insulin secretion (40 ± 16%; $P = 0.020$). No additive action regarding either AA formation or insulin secretion was seen by combining TPA overnight and ACA, which implies the involvement of an additional PLA_2- and PKC-independent signaling mechanism. The results show that CCK-8, in contrast to carbachol, activates Ca^{2+}-independent PLA_2 in islets and that the PLA_2-activating capacity of CCK-8 is partly PKC dependent. Hence, Ca^{2+}-independent PLA_2 seems important for the insulinotropic effect of CCK-8, but not for that of carbachol. *Diabetes* 47:1436–1443, 1998

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Received for publication 16 July 1997 and accepted in revised form 26 May 1998.

AA, arachidonic acid; ACA, *p*-amylcinnamoylantranilic acid; CCK-8, cholecystokinin-8; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; RHC 80267, 1,6-biscyclohexylideneaminooxycarbonylamino-hexane; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

Besides circulating nutrients, insulin secretion is affected by a variety of hormones and neuropeptides (1,2). The role of these peptides in islet physiology as well as their putative involvement in the regulation of insulin release in states of type 2 diabetes is largely unknown. Cholecystokinin is a gastrointestinal hormone released during meal intake (3), and also a pancreatic neuropeptide (4). Its COOH-terminal octapeptide (cholecystokinin-8 [CCK-8]) has been shown to activate specific CCK receptors on the β-cell (5) and to stimulate insulin secretion (6). CCK-8 is released into the circulation upon food intake and has been suggested to participate physiologically in the regulation of postprandial insulin secretion (7). The postprandial release of CCK-8 has been shown to be reduced in patients with type 2 diabetes, and the concomitant rise in blood glucose has been shown to be abolished by CCK-8 infusion (8). A putative role for CCK-8 in type 2 diabetes has been further underlined by studies in different animal models, which show a coupling between type 2 diabetes predisposition and insensitivity to CCK-8 stimulation (9) and an enhanced compensatory β-cell responsiveness to CCK-8 during type 2 diabetes development (10). Because CCK-8 thus seems to be a physiological regulator of insulin secretion with a possible involvement in the pathophysiology of type 2 diabetes, its mechanisms of action in the β-cell are important to delineate.

In islets, CCK-8 is known to stimulate phospholipase C (PLC) (11,12) to release Ca^{2+} from intracellular Ca^{2+} stores (13) and to activate protein kinase C (PKC) (14). However, we presented results implying that this pathway is not the sole signaling mechanism underlying CCK-8–stimulated insulin secretion, because PKC downregulation only partially reduces CCK-8–stimulated insulin secretion (14). Instead, we suggested that phospholipase A_2 (PLA_2) is also of importance for CCK-8–stimulated insulin secretion, because *p*-amylcinnamoylantranilic acid (ACA), which is a specific PLA_2 inhibitor (15), diminished CCK-8–stimulated insulin secretion from isolated rat islets (16). A role for PLA_2 in CCK-8–stimulated insulin secretion is further supported by the finding that the peptide promoted efflux of [^{3}H]arachidonic acid (AA) from prelabeled islets, which reflects PLA_2 activity (17), concomitantly with its stimulation of insulin secretion (16). Therefore, analogous to the studies on CCK-8–induced amylase secretion in rat pancreatic acini (18), activation of PLA_2

and subsequent formation of AA might also be of importance for the insulinotropic effect of CCK-8.

PLA_2 activation with formation of AA has previously been proposed as an important intracellular signaling pathway that mediates a variety of regulatory influences in several different cell systems (19,20). PLA_2 has been shown to be present within both human and rat islets (21–23), where it is assumed to exist in a Ca^{2+}-dependent (24) and a Ca^{2+}-independent ATP-stimulatable (25) form, both participating in the glucose-dependent insulin secreting process (26,27). Ca^{2+}-independent ATP-stimulatable PLA_2 has been proposed as an alternative way for glucose metabolism to generate insulin secretion when only small increments in ATP production are possible (27). It is not known, however, to what extent this PLA_2 form is sensitive to CCK-8 stimulation and whether it differs from stimulation by the cholinergic agonist carbachol, which, like CCK-8, is known to activate PLA_2 in islets (17). Neither is it known whether there is an interrelationship between CCK-8–stimulated PLA_2 activation and the signaling pathway involving PKC, which is of importance for CCK-8–stimulated insulin release (7).

Therefore, in the present study, we have examined the ability of CCK-8 to stimulate Ca^{2+}-independent PLA_2 in isolated rat islets compared with the ability of carbachol. To investigate the contribution of PLA_2, we examined AA efflux, lysophosphatidylcholine accumulation, and insulin secretion from prelabeled islets in the presence and absence of the PLA_2 inhibitor ACA (15) and the diglyceride lipase inhibitor RHC 80267 (1,6-biscyclohexylideneaminooxycarbonylamino-hexane) (17). The dependence on extracellular Ca^{2+} influx was studied by using the Ca^{2+} channel inhibitor verapamil (17) and by removal of extracellular Ca^{2+}, whereas the dependence on intracellular Ca^{2+} stores was investigated by the use of the Ca^{2+}-ATPase inhibitor thapsigargin (28). Furthermore, the possible interrelationship between CCK-8–induced PLA_2 activation and PKC was examined by studying islets subjected to PKC downregulation induced by an overnight incubation with the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (29).

RESEARCH DESIGN AND METHODS

Animals. The experiments were performed on pancreatic islets isolated from male Sprague-Dawley rats (Möllegaard A/S, Ll. Skensved, Denmark), weighing 200–300 g, with free access to a standard pellet diet and tap water.

Isolation and incubation of islets. Islets were isolated by the collagenase digestion technique (30). In brief, the common bile duct was cannulated and ligated at the papilla Vateri. The pancreas was filled through the cannula with 10 ml of cold Hanks' balanced salt solution (Sigma, St. Louis, MO), supplemented with 1.0 mg/ml collagenase P (activity: 1.52 U/mg; Boehringer Mannheim, Mannheim, Germany). The pancreas was then removed and incubated for 24 min at 37°C. After being rinsed in Hanks' solution, selected islets were hand-picked under a stereomicroscope and cultured overnight in 10 ml of RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2.05 mmol/l L-glutamine, 2.5 µg/ml amphotericin B (all GIBCO BRL, Paisley, Scotland), 100 IU/ml penicillin, and 100 µg/ml streptomycin (both Biological Industries, Beit Haemek, Israel) at 37°C (pH 7.4) in a humid atmosphere of 95% air and 5% CO_2. After one night's incubation, the islets were rinsed in a modified HEPES medium (pH 7.4) consisting of 125 mmol/l NaCl, 5.9 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 1.28 mmol/l $CaCl_2$, and 25 mmol/l HEPES (Boehringer Mannheim), supplemented with 0.1% human serum albumin (Behringwerke, Marburg, Germany) and 3.3 mmol/l glucose (Fluka Chemie AG, Buchs, Switzerland). The islets were then pre-incubated for 30 or 60 min in the modified HEPES medium at 3.3 mmol/l glucose in an atmosphere of 5% CO_2 at 37°C (pH 7.4). Thereafter, groups of islets were transferred into separate chambers for 30 or 60 min of incubation in air equilibrated with 5% CO_2 at 37°C (pH 7.4) in the HEPES medium supplemented with 0.1% human serum albumin, with or without addition of synthetic sulfated CCK-8 (the COOH-terminal octapeptide of cholecystokinin; $CCK_{26–33}$; 100 nmol/l; Sigma), carbachol (100 µmol/l; BDH Chemicals, Poole, England, U.K.), and different inhibitors. The glucose concentration in the medium was 5.6 mmol/l, which previously has been shown to promote a maximal insulin-releasing effect of CCK-8 (16). The HEPES medium was continuously gassed in an atmosphere of 95% air and 5% CO_2.

Induction of PKC downregulation. Isolated islets were incubated overnight at 37°C (pH 7.4) in an atmosphere of 5% CO_2 in 10 ml of RPMI 1640 (complete as above) in the presence of the phorbol ester TPA (500 nmol/l; Sigma). Prolonged exposure to TPA has previously been shown to downregulate PKC activity in pancreatic islets (28). The control islets were incubated overnight with the phorbol ester 4α-phorbol 12,13-didecanoate (Sigma; 500 nmol/l), which is without activity regarding PKC (29).

Incubation of [^{3}H]AA-labeled islets. Islets, isolated as above, were incubated overnight in 10 ml of RPMI 1640 (complete as above) supplemented with 4 µCi of [^{3}H]AA (specific activity: 100 Ci/mmol; Du Pont-NEN, Boston, MA) at 37°C (pH 7.4) in an atmosphere of 95% air and 5% CO_2. After the overnight incubation in [^{3}H]AA, the islets were pre-incubated for 30 min and then transferred in groups of 25 into separate chambers, where they were incubated for another 30 min in 1 ml of HEPES medium supplemented with 0.1% human serum albumin and 5.6 mmol/l glucose at 37°C (pH 7.4) in air equilibrated with 5% CO_2, with or without the addition of CCK-8 (100 nmol/l), carbachol (100 µmol/l), ACA (50 µmol/l; Calbiochem, La Jolla, CA), RHC 80267 (1,6-biscyclohexylideneaminooxycarbonylamino-hexane; 35 µmol/l; Calbiochem), thapsigargin (1 µmol/l; Sigma), and verapamil (100 µmol/l; Sigma). The studies performed after removal of extracellular Ca^{2+} were executed as described above, with the exception that the incubation medium, both during the pre-incubation and the experimental incubation, was composed without $CaCl_2$ and supplemented with 0.1 mmol/l of the calcium chelator EGTA (Sigma). After the incubation, 500 µl of the medium surrounding the islets was taken from each chamber, and the radioactivity was determined by liquid scintillation counting using the scintillation cocktail Optiphase (Wallac Oy, Turki, Finland). The 25 islets were retrieved as well, and their radioactivity was determined. Then, the radioactivity in the medium removed was expressed as a percentage of the total islet radioactivity. Another 25 µl of the medium was removed and frozen at –18°C until it was analyzed for its content of insulin.

Extraction of AA. AA was extracted as previously described by Hou et al. (31). After the overnight incubation in [^{3}H]AA, the islets were preincubated for 30 min. Thereafter, groups of 50 islets were incubated for another 30 min in 1 ml of HEPES medium supplemented with 0.1% human serum albumin and 5.6 mmol/l glucose (37°C; pH 7.4; 95% air/5% CO_2), with or without addition of CCK-8 (100 nmol/l). After incubation, 750 µl of the medium of two separate chambers was retrieved and transferred to polypropylene tubes, thus containing 1.5 ml of the medium each. The tubes were frozen and stored at –18°C until thin-layer chromatography analysis of AA was performed. Before the extraction, 25 µl of nonlabeled AA (0.5 mg/ml; Sigma) was added to each tube to improve recovery. Then, 1.5 ml of methanol/choloroform/HCl (200:200:1 by volume) were added to the 1.5 ml medium. The tubes were vortex-mixed for 1 min, when the aqueous phase was re-extracted twice with 1.5 ml $CHCl_3$. The samples were centrifuged for 5 min at 4°C and 1,500 rpm after each extraction step. The lower organic phase of each extraction was transferred to a new polypropylene tube. Thereafter, the organic phase was concentrated under a steam of nitrogen and reconstituted in 55 µl of chloroform.

One-dimensional thin-layer chromatography analysis of AA. A sample (25 µl) from each tube was applied to 10 × 20 cm silica-gel thin-layer chromatography plates (Merck, Darmstadt, Germany). Another 25-µl sample was removed for detection of total radioactivity. The plates were developed with petroleum spirit 40–60/diethyl ether/acetic acid (60:45:1 by volume). AA was identified by an unlabeled AA standard and detected by I_2 vapor. The AA R_F value was 0.040. The curettage of the AA spot was examined for its content of radioactivity, which was determined by liquid scintillation counting. Radioactivity in each peak is expressed as a percentage of the total counts of the sample.

Extraction of lysophospholipids. Lysophospholipids were extracted as previously described by Konrad et al. (17). Islets, isolated as above, were incubated overnight in 10 ml of RPMI 1640 (complete as above) supplemented with 40 µCi of [^{3}H]palmitidic acid (specific activity: 51 Ci/mmol; Du Pont-NEN) at 37°C (pH 7.4) in an atmosphere of 95% air and 5% CO_2. After the overnight incubation in [^{3}H]palmitidic acid, the islets were pre-incubated for 30 min. Thereafter, groups of 50 islets were incubated for another 30 min in 1 ml of HEPES medium supplemented with 0.1% human serum albumin and 5.6 mmol/l glucose (37°C; pH 7.4; 95% air/5% CO_2), with or without addition of CCK-8 (100 nmol/l). After incubation, 50 islets and 250 µl of the medium of four separate chambers were retrieved and transferred to polypropylene tubes, thus containing 200 islets and 1 ml medium each. Two milliliters of ice-cold chloroform/methanol (1:2 by volume) supplemented with 0.25% of the antioxidant butylated hydroxytoluene was added. The tubes were then immediately chilled for 15 min in a dry ice/ethanol bath and stored at –18°C until the thin-layer chromatography of lysophospholipid analysis was performed. Before the thin-layer chromatography, 10 µl of a phospholipid standard mixture (Sigma) containing lysophosphatidylcholine (0.3 mg/ml), phosphatidylcholine (1.5

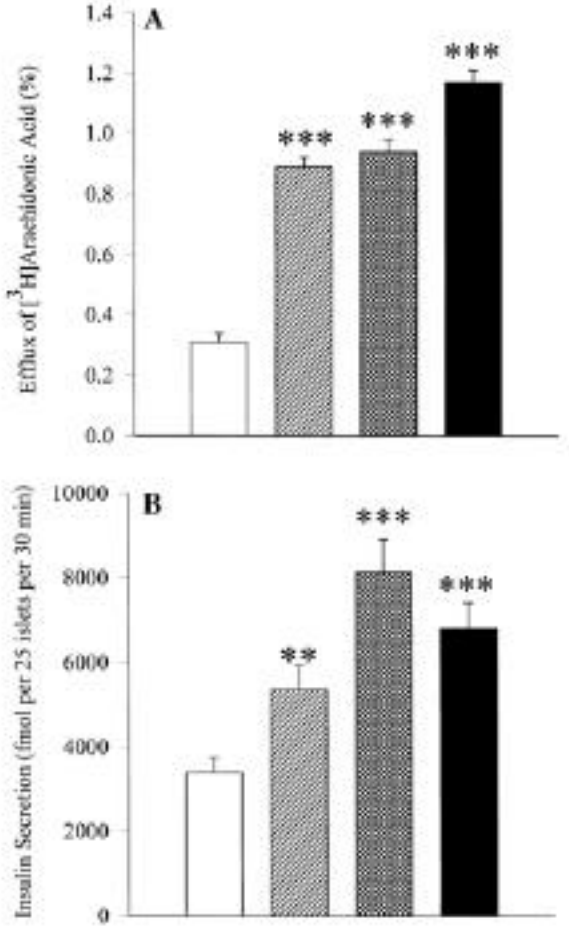

FIG. 1. Fractional efflux of [^{3}H]AA expressed as the percentage release of total incorporated radioactivity (*A*) and concomitant insulin secretion (*B*) from prelabeled isolated islets incubated for 30 min in the presence of glucose (5.6 mmol/l), with or without CCK-8 (100 nmol/l) and carbachol (100 µmol/l). n = 21, **P < 0.01, ***P < 0.001 versus control. □, control; ▨, CCK-8; ▩, carbachol; ■, CCK-8 and carbachol.

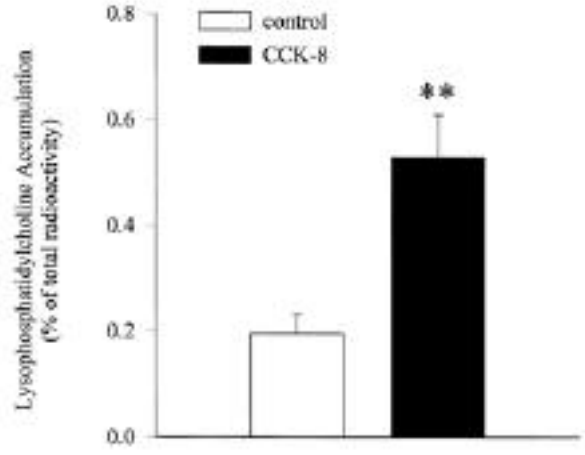

FIG. 2. Lysophosphatidylcholine accumulation expressed as the percentage ratio of total radioactivity from isolated islets prelabeled with [^{3}H]palmitidic acid and incubated for 30 min in the presence of glucose (5.6 mmol/l), with or without CCK-8 (100 nmol/l). n = 4–6, **P < 0.01 versus control.

mg/ml), phosphatidylethanolamine (1.2 mg/ml), and phosphatidylinositol (0.9 mg/ml) were added to each tube to aid in recovery, followed by 1 ml chloroform. The tubes were then vortex-mixed for 1 min, sonicated for 30 min, vortex-mixed for another 1 min, and centrifuged for 15 min at 4°C and 800*g*. Then, the lower organic phase was transferred to a polypropylene tube. The upper aqueous phase was re-extracted twice with 1 ml chloroform, whereupon the extracts were combined with the previously transferred organic phase. After that, the organic phase was washed with 1 ml of water, concentrated twice under a steam of nitrogen, and reconstituted in 25 µl of chloroform containing the above-mentioned standard lipids, to visualize the spots on the two-dimensional thin-layer chromatography.

Two-dimensional thin-layer chromatography analysis of lysophospholipids. A sample (10 µl) from each tube was applied to 10 × 10 cm silica-gel thin-layer chromatography plates (Merck). Another 10 µl sample was removed for detection of total radioactivity. Then, the plates were developed in the first dimension with chloroform/methanol/28% NH_4OH (130:70:11 by volume). After being carefully dried, the plates were developed in the second dimension with chloroform/methanol/formic acid/water (55:28:5:1 by volume). Radioactivity in each peak is expressed as a percentage of the total counts of the sample. The identity of the peaks was assigned by comparison with the unlabeled phospholipid standards. The lysophosphatidylcholine R_F value was 0.04 in the first dimension and 0.07 in the second dimension.

Analysis of insulin. After incubation, 25 µl of the medium surrounding the islets were removed and frozen at –18°C for subsequent analysis of insulin. The analysis was performed radioimmunochemically, using a guinea pig anti-rat insulin antibody, mono ^{125}I-labeled human insulin as a tracer and rat insulin as standard (Linco Research, St. Charles, MO). For the separation of free and bound radioactivity, the double antibody technique was used.

Statistical analysis. All values are presented as means ± SE. Student's t test for unpaired data was used for statistical evaluation. A P value of P < 0.05 was considered significant.

RESULTS

Effect of CCK-8 and carbachol on [^{3}H]AA efflux, lysophosphatidylcholine accumulation, and insulin secretion. To establish the involvement of PLA_2 in CCK-8–stimulated and carbachol-stimulated insulin secretion, we examined the efflux of radioactivity and the concomitant insulin secretion during a 30-min incubation period from islets prelabeled with [^{3}H]AA (Fig. 1). We found that CCK-8 (100 nmol/l) increased efflux of [^{3}H]AA by 190 ± 13% (P < 0.001; n = 21) and insulin secretion by 58 ± 20% (P = 0.006; n = 21). To confirm that the detected radioactivity was indeed [^{3}H]AA, its nature was characterized by thin-layer chromatography. We found that 60 ± 9% of the radioactivity released from the islets was detected at the AA band on the thin-layer chromatography plate. We also found that the amount of AA compared with controls increased by 309 ± 49% after CCK-8 stimulation (P < 0.001; n = 5). To further establish the involvement of PLA_2, we also examined the effect of CCK-8 on lysophosphatidylcholine accumulation from islets prelabeled with [^{3}H]palmitidic acid (Fig. 2). Thereby, we found that CCK-8 (100 nmol/l) increased lysophosphatidylcholine accumulation by 170 ± 39% (P = 0.003; n = 4–6).

Furthermore, carbachol (100 µmol/l) enhanced efflux of [^{3}H]AA by 203 ± 16% (P < 0.001; n = 21) and insulin secretion by 140 ± 21% (P < 0.001; n = 21). CCK-8 and carbachol in combination increased [^{3}H]AA efflux by 277 ± 15% (P < 0.001; n = 21) and insulin secretion by 101 ± 21% (P < 0.001; n = 21). The effect on [^{3}H]AA efflux due to the combination of carbachol and CCK-8 was additive when compared with the effect of either agonist alone. Thus, [^{3}H]AA efflux after CCK-8 plus carbachol was 24 ± 6% higher than the effect of CCK-8 alone (P < 0.001; n = 21) and 31 ± 6% higher than the effect of carbachol alone (P < 0.001; n = 21). In contrast, regarding insulin secretion, no additional effect was seen by combining CCK-8 and carbachol (NS; n = 21). Thus, both CCK-8 and carbachol are able to activate PLA_2 in rat islets.

Effect of Ca^{2+} channel inhibition on CCK-8–induced and carbachol-induced [^{3}H]AA efflux and insulin secretion. To study whether PLA_2 activation due to CCK-8 and carbachol was dependent on influx of extracellular Ca^{2+}, we studied the

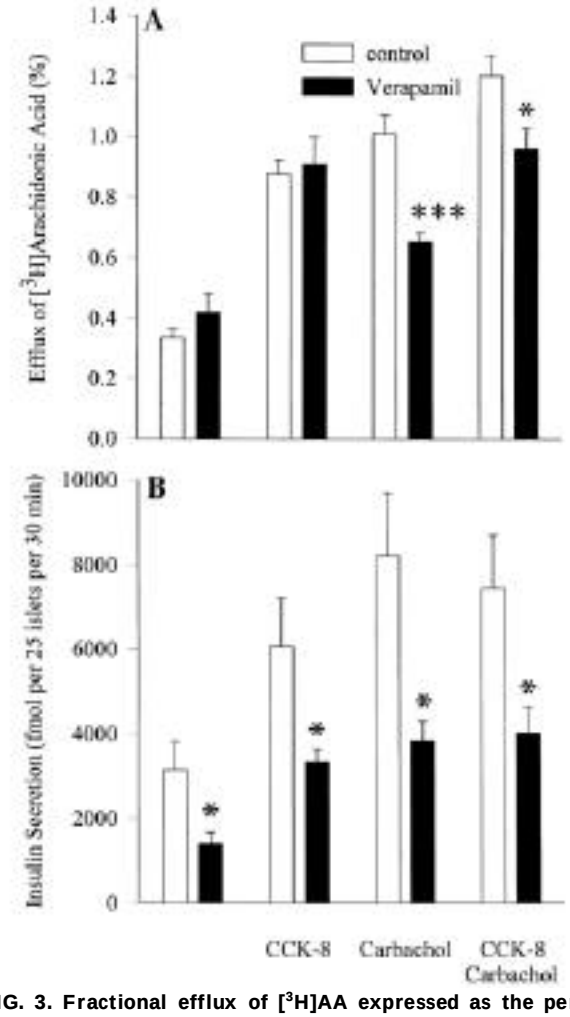

FIG. 3. Fractional efflux of [^{3}H]AA expressed as the percentage release of total incorporated radioactivity (*A*) and concomitant insulin secretion (*B*) from prelabeled isolated islets incubated for 30 min in the presence of glucose (5.6 mmol/l), with or without CCK-8 (100 nmol/l), carbachol (100 µmol/l), and verapamil (100 µmol/l). *n* = 9, **P* < 0.05, ****P* < 0.001 versus respective controls.

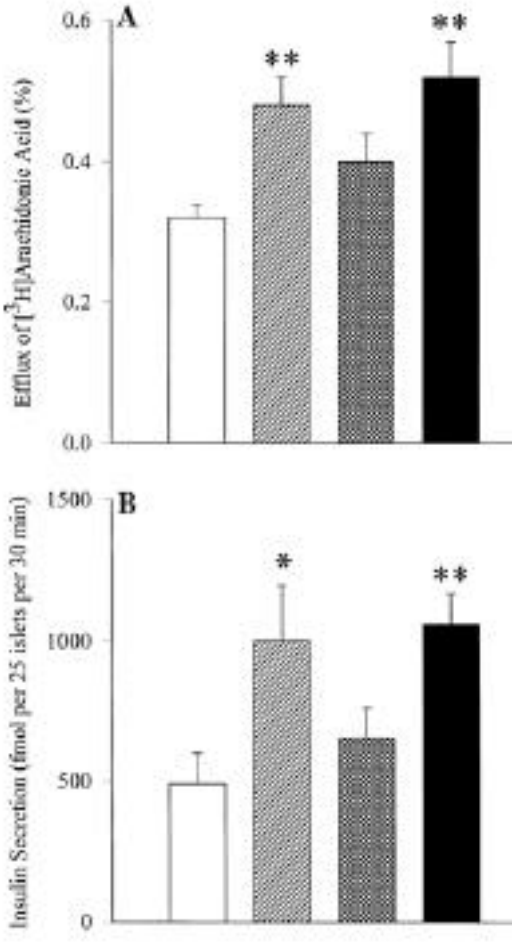

FIG. 4. Fractional efflux of [^{3}H]AA expressed as the percentage release of total incorporated radioactivity (*A*) and concomitant insulin secretion (*B*) from prelabeled isolated islets incubated for 30 min in a calcium-deficient medium in the presence of glucose (5.6 mmol/l), with or without CCK-8 (100 nmol/l) and carbachol (100 µmol/l). *n* = 6, **P* < 0.05, ***P* < 0.01 versus respective controls. □, control; ▨, CCK-8; ▩, carbachol; ■, CCK-8 and carbachol.

efflux of [^{3}H]AA and the concomitant insulin secretion from prelabeled islets incubated for 30 min together with the Ca^{2+} channel inhibitor verapamil (100 µmol/l; Fig. 3). It was found that the efflux of [^{3}H]AA induced by CCK-8 (100 nmol/l) remained unchanged (NS; n = 9). In contrast, the efflux induced by the cholinergic agonist carbachol (100 µmol/l) was, in the presence of 5.6 mmol/l glucose, reduced by 36 ± 7% by verapamil (P < 0.001; n = 9). This was equivalent to a reduction by 66 ± 12% of the net carbachol-stimulated efflux (i.e., after subtracting efflux values of control islets incubated with glucose alone). CCK-8–mediated insulin secretion was reduced by 45 ± 20% (P = 0.036; n = 9), and carbachol-mediated insulin secretion was reduced by 53 ± 19% (P = 0.013; n = 9) by verapamil. When CCK-8 and carbachol were combined, efflux of [^{3}H]AA was diminished by 20 ± 8% (P = 0.021; n = 9) and insulin secretion was diminished by 54 ± 19% (P = 0.027; n = 9). Thus, Ca^{2+} channel blockade by verapamil reduces carbachol- but not CCK-8–induced PLA_2 activation.

Effect of Ca^{2+} omission on CCK-8– and carbachol-induced [^{3}H]AA efflux and insulin secretion. To further investigate if PLA_2 activation due to CCK-8 and carbachol was dependent on uptake of extracellular Ca^{2+}, we studied the efflux of [^{3}H]AA and the concomitant insulin secretion from prelabeled islets incubated for 30 min in a medium where Ca^{2+} was removed (Fig. 4). Although CCK-8–mediated [^{3}H]AA efflux and insulin secretion under these conditions were reduced by 43 ± 7% (P < 0.001; n = 6) and 78 ± 14% (P < 0.001; n = 6), respectively, CCK-8 still increased [^{3}H]AA efflux by 48 ± 14% (P = 0.006; n = 6) and insulin secretion by 105 ± 46% (P = 0.025; n = 6) compared with controls. In contrast, in the absence of extracellular Ca^{2+}, carbachol was unable to alter either [^{3}H]AA efflux or insulin secretion (NS; n = 6). When combining CCK-8 and carbachol in the absence of extracellular Ca^{2+}, no additional effect was seen when compared with the effect of the separate agonists alone, either for [^{3}H]AA efflux (NS; n = 6) or insulin secretion (NS; n = 6). Thus, omission of extracellular Ca^{2+} inhibits carbachol- but not CCK-8–induced PLA_2 activation.

Effect of intracellular Ca^{2+} store depletion on CCK-8–induced and carbachol-induced [^{3}H]AA efflux and insulin secretion. To examine whether PLA_2 activation due to CCK-8 and carbachol was dependent on release of Ca^{2+}

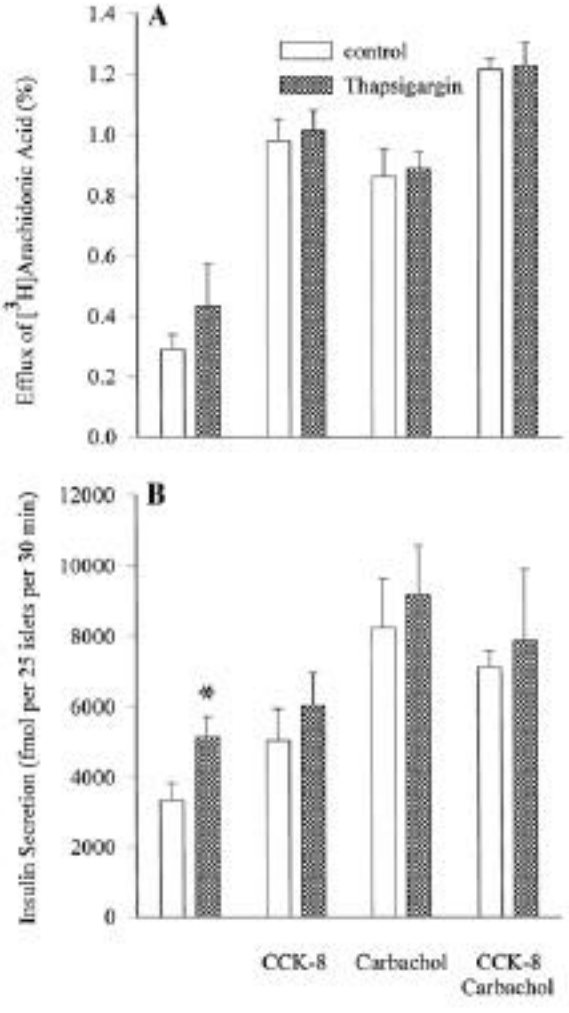

FIG. 5. Fractional efflux of [^{3}H]AA expressed as the percentage release of total incorporated radioactivity (*A*) and concomitant insulin secretion (*B*) from prelabeled isolated islets incubated for 30 min in the presence of glucose (5.6 mmol/l), with or without CCK-8 (100 nmol/l), carbachol (100 µmol/l), and thapsigargin (1 µmol/l). n = 6, *P < 0.05.

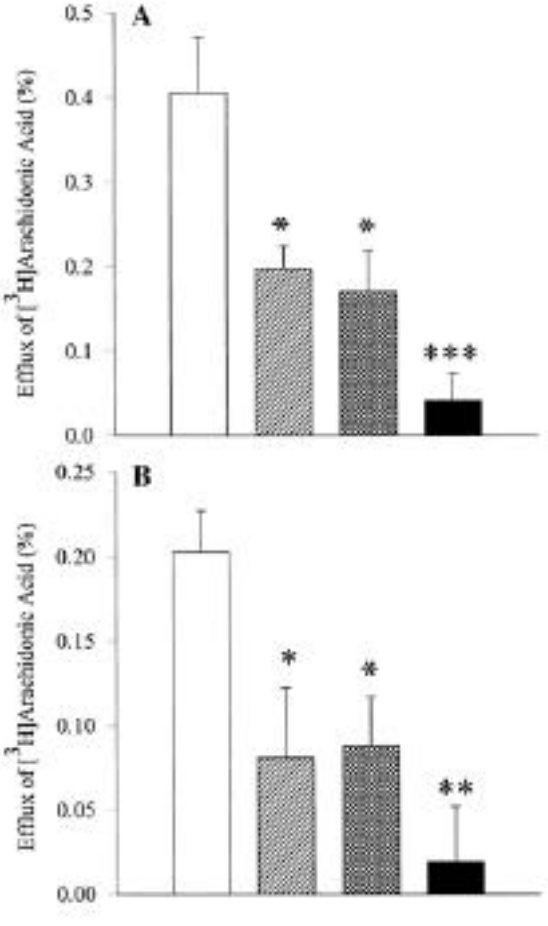

FIG. 6. Fractional efflux of [^{3}H]AA expressed as the percentage release of total incorporated radioactivity from prelabeled isolated islets incubated for 30 min in the presence of glucose (5.6 mmol/l), with or without CCK-8 (100 nmol/l), RHC 80267 (35 µmol/l), and ACA (50 µmol/l), in the presence (*A*) or absence (*B*) of extracellular Ca^{2+}. The calculated net effects of CCK-8 are shown after subtracting values of control islets incubated with 5.6 mmol/l glucose alone from the values of islets incubated with glucose and CCK-8 together. n = 6, *P < 0.05, **P < 0.01, ***P < 0.001 versus controls. □, CCK-8; ▨, CCK-8 + RHC 80267; ▩, CCK-8 + ACA ; ■, CCK-8 + RHC 80267 + ACA.

from intracellular Ca^{2+} stores, we studied the efflux of [^{3}H]AA and the concomitant insulin secretion from prelabeled islets incubated together with the Ca^{2+}-ATPase inhibitor thapsigargin (1 µmol/l), which induces depletion of intracellular Ca^{2+} stores (Fig. 5). However, during a 30-min incubation period, efflux of [^{3}H]AA and concomitant insulin secretion because of the two secretagogues, alone or in combination, remained unaltered compared with controls (NS; n = 6). Thus, depletion of intracellular Ca^{2+} stores does not affect CCK-8– or carbachol-induced PLA_2 activation.

Effect of diglyceride lipase inhibition and PLA_2 inhibition on CCK-8–induced [^{3}H]AA efflux in the presence and absence of extracellular Ca^{2+}. To delineate to what extent CCK-8–stimulated AA generation is dependent on PLA_2 activation versus diglyceride lipase activation, we measured the efflux of [^{3}H]AA and from prelabeled islets during a 30-min incubation period in the presence of the diglyceride lipase inhibitor RHC 80267 (35 µmol/l) and the PLA_2 inhibitor ACA (50 µmol/l; Fig. 6*A*). After subtracting values of control islets incubated with 5.6 mmol/l glucose alone from the values of islets incubated with glucose and CCK-8 together, we found that RHC 80267 diminished the net CCK-8–induced efflux of [^{3}H]AA by 51 ± 17% (P = 0.014; n = 6). Furthermore, ACA reduced the net CCK-8–stimulated efflux of [^{3}H]AA by 58 ± 20% (P = 0.016; n = 6). When combining RHC 80267 and ACA, the net CCK-8–induced efflux of [^{3}H]AA was decreased by 90 ± 18% (P = 0.001; n = 6). To study the role of extracellular Ca^{2+} during the involvement of PLA_2 and diglyceride lipase, we also performed the experiments with ACA and RHC 80267 in a Ca^{2+}-deficient environment (Fig. 6*B*). Then, after subtracting controls incubated with 5.6 mmol/l glucose alone, we found that RHC 80267 (35 µmol/l) diminished the net CCK-8–induced efflux of [^{3}H]AA by 58 ± 25% (P = 0.041; n = 6). Furthermore, ACA (50 µmol/l) reduced the net CCK-8–stimulated efflux of [^{3}H]AA by 55 ± 20% (P = 0.020; n = 6). When RHC 80267 and ACA were combined, the net CCK-8–induced efflux of [^{3}H]AA was decreased by 90 ± 21% (P = 0.002; n = 6). Thus, CCK-8–induced AA generation and insulin secretion are partly dependent on diglyceride lipase activation and partly dependent on PLA_2 activation in both the presence and absence of extracellular Ca^{2+}.

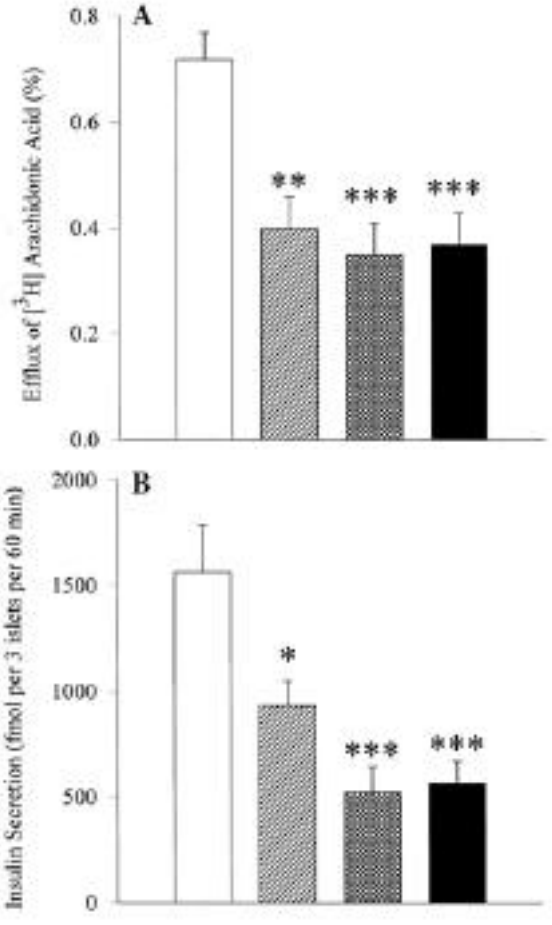

FIG. 7. Fractional efflux of [^{3}H]AA expressed as the percentage release of total incorporated radioactivity from prelabeled isolated islets incubated for 30 min (*A*) and insulin release from prelabeled isolated islets incubated for 60 min (*B*) in the presence of glucose (5.6 mmol/l) and CCK-8 (100 nmol/l), with or without the PLA_2 inhibitor ACA (50 µmol/l), in islets subjected or not subjected to PKC downregulation by an overnight incubation with TPA. The calculated net effects of CCK-8 are shown after subtracting values of control islets incubated with 5.6 mmol/l glucose alone from the values of islets incubated with glucose and CCK-8 together. For *A*, $n = 6$; for *B*, $n = 13–16$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus controls. □, CCK-8; ▨, CCK-8 + TPA overnight; ▩, CCK-8 + ACA ; ■, CCK-8 + TPA overnight + ACA.

Effect of PKC downregulation and PLA_2 inhibition on CCK-8–induced [^{3}H]AA efflux and insulin secretion. To examine the PKC dependence of CCK-8–stimulated PLA_2 activation, we studied the efflux of [^{3}H]AA from prelabeled islets during a 30-min incubation period (Fig. 7*A*) and the insulin secretion from unlabeled islets during a 60-min incubation period (Fig. 7*B*), after inducing PKC downregulation through an overnight incubation with the phorbol ester TPA (500 nmol/l). After subtracting values of control islets incubated with 5.6 mmol/l glucose alone from the values of islets incubated with glucose and CCK-8 together, we found that overnight incubation with TPA reduced the net CCK-8–induced efflux of [^{3}H]AA by 45 ± 12% ($P = 0.003$; $n = 6$) and the insulin secretion by 40 ± 16% ($P = 0.020$; $n = 14–16$). Furthermore, the PLA_2 inhibitor ACA (50 µmol/l) diminished the net CCK-8–stimulated efflux of [^{3}H]AA by 52 ± 11% ($P = 0.001$; $n = 6$) and the insulin secretion by 67 ± 16% ($P < 0.001$; $n = 13–16$). When overnight incubation with TPA and ACA were combined, no further inhibition of the net effects of CCK-8 was seen regarding either [^{3}H]AA efflux (NS; total diminution by 49 ± 11%; $P = 0.001$; $n = 6$) or insulin secretion (NS; total reduction by 64 ± 16%; $P = 0.001$; $n = 16$). Thus, CCK-8–induced PLA_2 activation is partly dependent on PKC.

DISCUSSION

The results of this study imply that both CCK-8 and carbachol activate PLA_2 in rat islets. Our results further indicate that PLA_2 activation due to CCK-8 stimulation is independent of both influx of extracellular Ca^{2+} and liberation of intracellular Ca^{2+}. This was in contrast to the PLA_2-stimulating effect of carbachol, which was dependent on extracellular Ca^{2+} influx. Moreover, the results suggest that the CCK-8–induced PLA_2 activation is partly dependent on PKC stimulation, even though a considerable share of the CCK-8–mediated PLA_2 activation seems to occur independently of PKC.

Earlier studies have shown that PLA_2 can be divided into two different subtypes: the secretory (extracellular, low molecular mass) form and the cytosolic (intracellular, high molecular mass) form (32,33). The cytosolic PLA_2, present in human and rat islets (21–23), hydrolyses plasma membrane phospholipids after having been translocated to the plasma membrane upon cellular activation through a process involving the binding of Ca^{2+} (34). However, during recent years, it has been apparent that PLA_2 can also be activated independently of Ca^{2+}, i.e., during Ca^{2+} channel blockade or after removal of extracellular Ca^{2+} (35,36). In addition, the Ca^{2+}-independent PLA_2 activity has been described to exist in substantial amounts within rat and human islets and has been shown to be activated by glucose metabolism–generated ATP (25). Its importance is further underscored by findings that when the Ca^{2+}-independent PLA_2 is pharmacologically inhibited, the glucose-induced stimulation of insulin secretion is severely suppressed (27). In fact, it has been suggested that this ATP-stimulatable Ca^{2+}-independent PLA_2 is required for optimal glucose responsiveness, because it has been shown that clonal insulinoma cell lines, which express ATP-stimulatable Ca^{2+}-independent PLA_2 activity, exhibit glucose responsiveness, whereas other insulinoma cell lines, which do not express ATP-stimulatable Ca^{2+}-independent PLA_2 activity, are in general unresponsive to glucose (37).

Whether CCK-8 stimulates a Ca^{2+}-dependent or a Ca^{2+}-independent form of islet PLA_2, or perhaps both, as well as whether the mechanism of CCK-8 in this regard is different from that of the cholinergic agonist carbachol, is unknown and was therefore examined. The study was methodologically based on three different approaches, all known to reflect the involvement of PLA_2. First, we delineated the efflux of [^{3}H]AA from prelabeled islets, which is a technique shown by Konrad et al. (17) to indirectly determine PLA_2 activity. Second, we detected lysophosphatidylcholine accumulation, which also reflects PLA_2 activity (17). Third, we induced PLA_2 inhibition by the PLA_2 inhibitor ACA at a concentration that previously has been shown to inhibit glucose-induced AA accumulation in rat islets without disturbing glucose metabolism or affecting PLC activity (15).

The results of all three approaches show that CCK-8 stimulates PLA_2. However, the results also imply that the AA generation induced by CCK-8 does not solely reflect PLA_2 activity, but also the sequential actions of PLC and diglyceride

lipase, which is a well-known pathway mediating AA generation in islets (38). In fact, the results indicate that the PLA_2 pathway and the PLC/diglyceride lipase pathway are almost equally responsible for the CCK-8–mediated AA formation, and that the effects of these two pathways are additive. However, cautious interpretations should be made when quantifying the role of the two separate pathways, because the inhibitors used have been suspected to be not entirely specific. Thus, it was recently shown that both RHC 80267 and ACA, concomitantly with their inhibitory action on insulin secretion, increased $^{86}Rb^+$ efflux from prelabeled mouse islets, although in this respect the effect of ACA was weak (39). This indicates that besides their inhibitory action on AA accumulation (15,17), RHC 80267 and, to a smaller extent, ACA, might open ATP-sensitive K^+-channels. Therefore, our results in this regard need to be further confirmed. The equal contribution of the PLA_2 and the PLC/diglyceride lipase pathways in our study was evident regardless of whether or not extracellular Ca^{2+} was present. This is interpreted to indicate that CCK-8 is able to exploit Ca^{2+}-independent actions of both PLA_2 and PLC/diglyceride lipase to accomplish AA generation. The other two PLA_2 subclasses besides the Ca^{2+}-independent form that are shown to be present in islets—the cytosolic form and the secretory form (21–23)—are known to require Ca^{2+} for their activation (33). Therefore, although a direct demonstration of ACA-induced inhibition of the Ca^{2+}-independent form of PLA_2 was not performed, our finding that ACA inhibits AA formation under Ca^{2+}-deficient conditions suggests that ACA inhibits the Ca^{2+}-independent form of PLA_2.

During Ca^{2+} channel blockade by verapamil, used at a concentration assumed to inhibit the entry of extracellular Ca^{2+} (17), CCK-8–induced efflux of [^{3}H]AA remained unchanged. In contrast, the efflux induced by carbachol was markedly reduced. Both agonists were used at dose levels that stimulate insulin secretion in rat islets (40,41). The findings are in accordance with our results obtained by removal of extracellular Ca^{2+}, implying that CCK-8, but not carbachol, induces activation of Ca^{2+}-independent PLA_2. Although the total amount of secreted insulin was considerably lower than in the presence of Ca^{2+}, CCK-8 was also able to stimulate insulin secretion in the absence of extracellular Ca^{2+}, whereas carbachol did not promote insulin release under these conditions. Thus, it seems as if the two potent AA generators CCK-8 and carbachol have a major dissimilarity in their modes of activating PLA_2. Such a hypothesis is further supported by our finding that the AA-generating effect of CCK-8 and carbachol in combination was significantly higher when compared with the effect of either of the two agents alone and by the fact that this additive effect was abolished when extracellular Ca^{2+} was omitted.

Because CCK-8 is known to liberate Ca^{2+} from intracellular stores in islets (13), we investigated if the PLA_2 activation induced by CCK-8 was dependent on release of Ca^{2+} from intracellular stores, by incubating islets together with thapsigargin. Thapsigargin at the concentration used inhibits the Ca^{2+}-ATPase pump activity of the endoplasmatic reticulum, which impairs the reabsorption of Ca^{2+} and depletes the Ca^{2+} content of the endoplasmatic reticulum (28). However, in spite of the presence of thapsigargin, the efflux of [^{3}H]AA and insulin secretion due to stimulation by CCK-8 or carbachol were unaffected, indicating that release of Ca^{2+} from intracellular stores is of no major importance for the activation of PLA_2 due to these two secretagogues. Thus, the ability of CCK-8 to activate PLA_2 seems independent of both extra- and intracellular Ca^{2+}, whereas the action of carbachol seems dependent on extracellular Ca^{2+}.

Ca^{2+}-independent PLA_2 activity has previously been shown to be directly stimulated by ATP (25). In fact, Ca^{2+}-independent PLA_2 has been proposed as an alternative way for glucose metabolism to generate insulin secretion when only small increments in ATP production are possible (27). Our results suggest that CCK-8, in this particular respect, might resemble ATP. Therefore, stimulation by CCK-8 would offer the β-cell an alternative possibility to activate Ca^{2+}-independent PLA_2 and accomplish Ca^{2+} influx and exocytosis under conditions of severely reduced ATP production, as, for example, under conditions of low glucose sensitivity such as during development of type 2 diabetes. CCK-8–mediated activation of Ca^{2+}-independent PLA_2 might therefore be an alternative rather than an additional mode to induce insulin secretion. Such a role is in line with our results that the combined effect of CCK-8 plus carbachol is additive regarding AA generation but not regarding insulin secretion. If this action of CCK-8 is preserved and perhaps exaggerated in type 2 diabetes, it could be a target for development of new drugs specifically activating β-cell CCK receptors. A previous clinical study indicating an important role for the insulinotropic effect of CCK-8 during type 2 diabetes might support such a theory (8).

The cellular events underlying CCK-8–induced PLA_2 activation have not been characterized. One possible constituent could be PKC, which previously has been proposed as a PLA_2 activator (32,42) and to be involved in CCK-8–induced insulin secretion (14). In the present study, we found that PKC downregulation, induced by an overnight incubation with TPA, reduced both CCK-8–induced efflux of [^{3}H]AA and insulin secretion, which suggests that PKC is important for the CCK-8–induced activation of PLA_2. However, a substantial part of the stimulating mechanism seems to be independent of PKC. Of further interest is the fact that when PKC downregulation and PLA_2 inhibition are combined, no additive reduction was seen regarding either the [^{3}H]AA efflux or the insulin secretion induced by CCK-8 when compared with the reduction caused by PLA_2 inhibition alone. This suggests that at least one additional PLA_2- and PKC-independent signaling pathway is involved in the AA-generating and insulinotropic effects of CCK-8.

In conclusion, the results of this study show that CCK-8–induced PLA_2 activation in islets also occurs after removal of extracellular Ca^{2+} and is insensitive to inhibition of plasma membrane Ca^{2+} channels as well as to depletion of intracellular Ca^{2+} stores, suggesting that CCK-8 has the ability to activate a Ca^{2+}-independent PLA_2. This indicates a dissimilarity between CCK-8 and the cholinergic agonist carbachol, which is unable to stimulate PLA_2 in the absence of extracellular Ca^{2+}. Furthermore, the results imply that the PLA_2-activating capacity of CCK-8 is partly dependent on PKC, even though a considerable share of the stimulating mechanism seems to be independent of PKC and, moreover, that CCK-8 is able to induce PLA_2 activation and insulin secretion despite inhibition of both PKC and PLA_2. These results suggest the involvement of least one additional signaling pathway for CCK-8 that is independent of both PLA_2 and PKC.

ACKNOWLEDGMENTS

This study was financially supported by the Swedish Medical Research Council (grant 14x-6834), Novo Nordic, Albert Påhlsson, Ernhold Lundström and Ester Ohlsson Foundations, Emma Ekstrand, Hildur Tegger and Jan Tegger Foundation, the Diabetes Association of Malmö with Surroundings, the Swedish Diabetes Association, Malmö University Hospital, and the Faculty of Medicine, Lund University.

The authors express their appreciation to Lilian Bengtsson and Lena Kvist for technical knowledge and to Ragnar Alm for substantial contribution in the thin-layer chromatography experiments.

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