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Dual Pathways for Carbamylcholine-Stimulated Arachidonic Acid Release in Rat Pancreatic Acini

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Recent studies suggested the involvement of arachidonic acid in the mediation of pancreatic amylase release. However, an effect of carbamylcholine on arachidonic acid release has not yet been reported in the exocrine pancreas. This study was performed to evaluate the effect of carbamylcholine on arachidonic acid release and determine the underlying intracellular mechanisms. From enzymatic assays, phospholipase A_2 and diacylglycerol lipase were activated by carbamylcholine and these activations were inhibited by the phospholipase A_2 inhibitors, mepacrine and aristolochic acid, and by the diacylglycerol lipase inhibitor RHC 80267. Carbamylcholine also increased arachidonic acid release in a concentration-dependent manner. Both phospholipase A_2 and diacylglycerol inhibitors partially inhibited carbamylcholine-stimulated arachidonic acid release. The phospholipase C inhibitor U73122 and the protein kinase C inhibitor staurosporine also caused partial inhibition. Arachidonic acid release by carbamylcholine was suppressed by the simultaneous addition of RHC 80267 with either phospholipase A_2 inhibitors. Our data demonstrate that phospholipase A_2 and diacylglycerol lipase are activated and arachidonic acid is released in pancreatic acini by carbamylcholine. Dual pathways are responsible for carbamylcholine-induced arachidonic acid release. One such pathway involves the sequential action of phospholipase C, protein kinase C and diacylglycerol lipase, whereas the other involves phospholipase A_2 activation.

Introduction

The muscarinic agonist carbamylcholine (Cch) binds to its receptor and causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), and the production of inositol trisphosphate (IP_3) and diacylglycerol (DAG). These two PIP_2 metabolites are respectively recognized to mobilize intracellular calcium and to activate protein kinase C (PKC) (Berridge, 1984; Cook and Wakelam, 1991). Calcium mobilization by Cch is also associated to pancreatic enzyme secretion (Yule and Williams, 1992).

Among second messengers formed through the activation of multiple known effectors, arachidonic acid (AA) has been recognized as an important factor (Noar, 1991). In the exocrine pancreas, AA release in response to two cholecystokinin (CCK) analogs, cerulein (Dixon and Hokin, 1984) and CCK-octapeptide (Pandol et al., 1991), has been clearly demonstrated and its involvement in enzyme secretion has been postulated. AA can be generated via two major signaling pathways, one of which being phospholipase A_2 (PLA_2) and the other the PIP_2 metabolite DAG, which on hydrolysis by DAG lipase, release AA (Irvine, 1982; Van den Bosch, 1980; Wang et al., 1994). In the exocrine pancreas, the source of AA has been identified from phospholipase C (PLC)-catalyzed breakdown of phosphatidylinositol followed by DAG lipase but not from PLA_2 acting on phosphatidylinositol (Dixon and Hokin, 1984). Another source seems to involve a phospholipase A action on phosphatidylcholine (Pandol et al., 1991). Among other reported pathways, AA could be generated from the activation of phospholipase A_1 followed by lysophospholipase B (Exton, 1990) or through the sequential activation of phospholipase D (PLD), phosphatidate phosphohydrolase (PPH) and di- and monoglyceride lipase (Martin and Wysolmerski, 1987). Although there are some reports describing AA release in response to cerulein (Dixon and Hokin, 1984) and CCK-octapeptide (Pandol et al., 1991) in the pancreas, and others on Cch-induced AA release from rat brain cortex membranes (Strosznajder and Samochocki, 1991), an effect of Cch on AA release from the exocrine pancreas has not been reported, nor any data on the intracellular signaling systems involved.

In the present studies, we, therefore, investigated the effect of Cch on AA release as well as the signal transduction pathways involved. These data indicate for the first time that Cch significantly activates PLA_2 and DAG lipase activities and stimulates AA release in pancreatic acinar cells. Two pathways are described as responsible for such

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Cch effects on AA release: one involves the sequential activation of PLC, PKC, and DAG lipase, whereas the other seems to implicate PLA_2 activation.

Materials and Methods

Materials

Carbamylcholine, bovine serum albumin (BSA, Fraction V and BSA fatty acid-free), soybean trypsin inhibitor type 2-S (SBTI), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), standards for thin layer chromatography (TLC), propranolol, 5,8,11,14-eicosatetraynoic acid (ETYA), arachidonic acid (5,8,11,14-eicosatetraenoic acid), aristolochic acid, mepacrine, sucrose, ethylenediamine tetraacetic acid (EDTA), ethyleneglycol-bis-(β-aminoethyl ether) N,N,N'N'-tetraacetic acid (EGTA), leupeptin, pepstatin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO). Phosphatidylethanol (PEt) was from Avanti Polar Lipids (Birmingham, AL). Purified collagenase (1424 U/mg) was from Worthington Biochemicals (Freehold, NJ). RHC-80267 was from Calbiochem (La Jolla, CA). Staurosporine was from Kyowa Hakko USA Inc. (New York, NY). U73122 was from Biomol (Plymouth Meeting, PA). Wortmannin was a gift from Sandoz, Canada. Silica gel G TLC plates (LK6D) were from Whatman (Clifton, NJ). Solvents for TLC and silica gel (28–200 mesh) were from Fisher (Pittsburgh, PA). [^{3}H] myristic acid (56 Ci/mmol) and 1-stearyl-2-[^{14}C] arachidonyl-glycerol (56 mCi/mmol) were from Amersham (Arlington Heights, IL). [^{3}H]-arachidonic acid (221 Ci/mmol) was from NEN. 1-palmitoyl-2-[^{14}C] arachidonoyl-phosphatidylcholine (53 mCi/mmol) was from Amersham Canada (Oakville, Ontario, Canada). Male Sprague-Dawley rats (200–240 g) were from our own colony.

Methods

Preparation of Pancreatic Acini

Pancreases from rats fasted overnight were removed and trimmed of fat and mesentery. A suspension of pancreatic acini was prepared as reported by Peikin (Peikin et al., 1978). Acini from five pancreases were resuspended in 32 mL of an enriched HEPES-buffered solution ([in m*M*] 24.1 HEPES, 98 NaCl, 6 KCl, 2.1 KH_2PO_4, 0.5 $CaCl_2$, 1.2 $MgCl_2$, 5 sodium pyruvate, 5 sodium fumarate, 5 sodium glutamate, and 11.4 glucose, as well as 0.01% [w/v] SBTI, 2.5% [v/v] glutamine, 1% [v/v] essential vitamin mixture, and 1% [v/v] BSA, adjusted to pH 7.4). For experiments with [^{3}H]-myristic acid incorporation, fatty acid-free BSA was used at concentration of 0.5% (w/v) in the same HEPES-buffered solution as described above. For experiments with [^{3}H]-AA incorporation, HEPES-buffered solution without BSA was used.

Uptake of [^{3}H]-AA into Pancreatic Acinar Cells

Acini from five pancreases were resuspended in 32 mL of HEPES-buffered solution and divided into flasks of 5 mL each. Acinar cells in each flask were incubated with [^{3}H]-AA (5 μCi/mL) for 120 min at 37°C. At the start of the incubation period (1 min) and every 30 min thereafter, 1 mL of acini suspension was removed from each flask followed by a 30 s centrifugation to discard the supernatant. The AA incorporation was ended by the addition of 2 mL of 5% trichloroacetic acid (TCA) to the pellets. The mixture of the acinar cells and TCA was vortexed vigorously and centrifuged again. The radioactivity present in the supernatant and in the TCA-precipitated materials was then determined after addition of scintillation fluid and expressed as percent of the total radioactivity present in acini.

DAG Lipase Assay

Cell extracts were prepared as described by Cybulsky (1991). Acini were pretreated with DAG lipase inhibitor RHC 80267 at 150 μ*M* in the appropriate groups for 15 min. A 15 min incubation followed in the presence or absence of 5 μ*M* Cch. At the end of the incubation, acini were centrifuged and the pellet obtained was washed twice with an homogenization buffer containing 50 m*M* HEPES, 0.25*M* sucrose, 1 m*M* EDTA, 1 m*M* EGTA, 20 μ*M* leupeptin, 20 μ*M* pepstatin, 0.1 m*M* PMSF, and 0.01% SBTI (w/v), pH 7.4. Acinar cells were disrupted in a glass-glass homogenizer and the homogenate was centrifuged at 1000*g* for 10 min to separate membrane components. The supernatant was collected and proteins were determined by the method of Bradford (1976). The assay of DAG lipase was adapted from Prescott and Majerus (1982). The substrate mixture of 1-stearyl-2-[^{14}C] arachidonyl-glycerol and unlabeled DAG were dried under nitrogen and suspended in 0.05% Triton X-100. After sonication for 20 s, 5-μL aliquot of the substrate mixture was distributed into tubes to give final 200 μ*M* concentration of DAG. The assay was initiated by adding the enzyme source containing 16 μg of protein and 7 m*M* $CaCl_2$ and terminated by adding ethanol containing 2% acetic acid. Incubation was carried out for 30 min at 37°C. Samples containing lipid standard were applied on thin layer chromatography plates. A solvent system consisting of hexane:diethyl ether:acetic acid (80:20:2) was used to separate AA which was identified as migrating with authentic standards detected using I_2 vapor. Areas containing AA were scraped and radioactivity was determined in a liquid scintillation counter. Results are expressed as DPM/mg protein.

Phospholipase A_2 Assay

PLA_2 assay was performed according to the method described by Jelsema (1987). After they were incubated with 5 μ*M* Cch for 15 min in the presence or absence of inhibitors, pancreatic acini were washed twice with the homogenization buffer containing 10 m*M* HEPES (pH 7.4), 1 m*M* EDTA, 1 m*M* EGTA, 0.34*M* sucrose, 10 μg/mL leupeptin, 0.01% SBTI (w/v) and 1 m*M* PMSF. After homogenization in a glass-glass homogenizer, nuclei and

debris were removed by centrifugation at 500*g* for 5 min. The low-speed supernates were spun at 20,000 rpm for 1 h. The supernates thus, obtained were assayed for protein content (Bradford, 1976) and for $cPLA_2$ activity using L-α-1-palmitoyl-2-[^{14}C-arachidonyl] phosphatidylcholine (^{14}C-PAPC) as substrate. Unlabeled dipalmitoyl phosphatidylcholine (0.08 mg/mL) was added to the radiolabeled ^{14}C-PAPC (10^7 dpm/mL) and the solvent (toluene:ethanol, 1:1) evaporated under N_2. The substrate mixtures were resuspended in 0.12*M* Tris-HCl, pH 8.8, and solubilized by sonication. They were incubated for at least 2 h at 37°C before use to allow reannealing. PLA_2 assay was then initiated by adding 20 μL of substrate, 75 μg of cytosolic protein in a reaction buffer, pH 8.8, containing 30 m*M* Tris-HCl, 5 m*M* $CaCl_2$, 40 m*M* $MgCl_2$, 0.6 m*M* NaCl, 4 m*M* glutathione in a total volume of 350 μL. Incubations were carried out at 37°C under slow agitation for 15 min. Reactions were stopped by addition of 1.25 mL of Dole's reagent (isopropanol:n-heptane:1 N H_2SO_4, 40:10:1). The enzymatically released ^{14}C-arachidonate was extracted by addition of 1.5 mL of n-heptane and 1.0 mL of H_2O. Samples were vortexed vigorously and centrifuged at 1000*g* for 10 min. 150 mg of silica gel (28–200 mesh, Fisher) were added to 1.5 mL of upper phase to remove the remaining radiolabeled substrate. Samples were vortexed, centrifuged at 1000*g* for 10 min, and the radioactivity of 1-mL aliquot of supernatant was determined in a scintillation counter after the addition of 10 mL of scintillation cocktail. PLA_2 activity was expressed as nmol of ^{14}C-AA released/mg of proteins/min. Background control values were subtracted.

Determination of AA Release

The method described by Sato was modified and used to measure AA (Sato et al., 1992). Pancreatic acini were labeled with [^{3}H]-AA (5 μCi/mL) for 90 min at 37°C in the HEPES-buffered solution without BSA. The labeled acini were washed three times with incubation buffer and resuspended in the incubation medium for an additional 30 min without the labeled AA. The acini were then incubated in the presence or absence of Cch at the indicated concentrations in the HEPES-buffered solution containing 0.5% (w/v) fatty acid-free BSA. ETYA was added to the incubation buffer prior to and during the stimulation to prevent AA from being further hydrolyzed into its metabolites and, therefore, facilitate observation of AA release. AA and other lipids were extracted separately from acini and from the incubation medium by adding methanol:chloroform:HCl (200:200:1, v/v/v) to the acini and to a 200 μL aliquot of the incubation medium. The mixture was well vortexed and the phases were separated by centrifugation after adding H_2O. The upper aqueous phase was discarded. The samples of the lower organic phase with standards added were dried under a stream of nitrogen, redissolved in 50 μL of chloroform, applied on TLC plates and developed in a solvent system consisting of petroleum ether/diethyl ether/acetic acid (60:45:1, v/v/v). AA was identified as migrating with an authentic AA standard detected by I_2 vapor. Areas containing AA were scraped and radioactivity was determined in a liquid scintillation counter. Intracellular AA was expressed as a percent of total radioactivity into the acini and extracellular AA as a percent of radioactivity in the phospholipids. Radioactivity in phospholipids was calculated by subtracting the radioactivity in intracellular AA from that of total radioactivity.

Determination of Phosphatidylethanol (PEt)

PEt was determined according to a method described previously (Rydzewska et al., 1993). After pancreatic acini were labeled with [^{3}H]-myristic acid (5 μCi/mL) for 1 h at 37°C in HEPES-buffered solution containing 0.5% fatty acid-free BSA and washed, they were incubated with or without CCh for 15 min in the medium containing 1.0% ethanol (v/v). At the end of incubation period, 1 mL of acini was removed and quickly centrifuged at 10,000*g* in a microcentrifuge for 15 s to separate the supernatant from the pellet. The supernatant was discarded. To the pellets, 2 mL of methanol:10 m*M* glycine (5:2, v/v) were added. To this methanol:glycine mixture, 2 mL of chloroform were added and mixed well. 1 mL of H_2O was then added to this mixture and the phases were separated by a 5-min centrifugation at 1000*g*. The upper aqueous phase was discarded. Standard samples of PEt were added into the organic phase, which was dried under nitrogen gas and spotted on TLC plates. The plates were developed in a solvent system containing chloroform:methanol:ammonium hydroxide (65:30:3, v/v/v). Areas containing PEt were scraped, and radioactivity was counted. PEt was normalized to the percent of total amount of the labeled lipids.

In all experiments where inhibitors of PLC, PLD, PLA_2, PKC, and DAG lipase were used, acini were always preincubated with each inhibitor for a 15-min period and then incubated for the indicated time periods in the presence or absence of CCh with the different inhibitors. These inhibitors were chosen to investigate potential enzymes involved in AA release. ETYA, a cyclo-oxygenase and lipoxygenase inhibitor (Turk et al., 1985), was used to limit AA degradation after its release. The PLC inhibitor U73122 (Yule and Williams, 1992), the PLD inhibitor wortmannin (Bonser et al., 1991), the PPH inhibitor propranolol (Sozzani et al., 1992) were used in combination with the DAG lipase inhibitor RHC 80267 (Sutherland and Amin, 1982) to distinguish AA generated from PLC and/or PLD activated pathways. Two PLA_2 inhibitors, mepacrine (Wallach and Brown, 1981) and aristolochic acid (Spangel et al., 1991) were also used to assess the involvement of PLA_2.

Data were analyzed by analysis of Variance (ANOVA) and Fisher and Scheffé tests. Significance was taken at the 5% level ($P < 0.05$).

Results

DAG Lipase and PLA_2 Activities

Enzymatic assays of DAG lipase and PLA_2 were performed using each enzyme-specific substrate to assess whether they could be activated by Cch and determine the validity of their respective inhibitor to modulate their activities. Table 1 shows that 5 μ*M* Cch significantly activated DAG lipase activity by 84% and that this activation was completely inhibited by the DAG lipase inhibitor RHC 80267 at 150 μ*M*. Sequential action of PLC-DAG lipase in AA release is suggested by the observation that the PLC inhibitor U73122 also effectively inhibited DAG lipase activity. In this system, it has to be remembered that the enzymes were stimulated by Cch in unbroken cells while the enzymatic assays were performed using homogenate supernatants. Also, inhibitors were present when the unbroken cells were stimulated. This precision explains why a PLC inhibitor can prevent DAG lipase activation. PLA_2 activity was substantially increased by 187% on stimulation by 5 μ*M* Cch. 150 μ*M* mepacrine and 200 μ*M* aristolochic acid totally inhibited the activation of PLA_2 by Cch. The PLC and DAG lipase inhibitors did not exhibit any inhibitory effect on Cch-induced PLA_2 activity (Table 1). Since we have clearly demonstrated that both DAG lipase and PLA_2 activities were activated by Cch and that these activations were inhibited by their respective enzyme inhibitors, these inhibitors were subsequently used as tools to further investigate the intracellular mechanisms involved in AA release.

Table 1
Effects of Carbachol on Pancreatic PLA_2 and DAG Lipase Activities

	PLA_2 activity nmol/mg protein/min	DAG lipase activity dpm/mg protein
Control	17.4 ± 0.8	60.1 ± 4.2
5 μ*M* Cch	49.9 ± 2.4*	110.5 ± 7.9*
5 μ*M* Cch + 150 μ*M* mepacrine	20.5 ± 1.5	
5 μ*M* Cch + 200 μ*M* aristolochic acid	19.9 ± 1.5	
5 μ*M* Cch + 10 μ*M* U731122	49.5 ± 3.0*	70.7 ± 5.4
5 μ*M* Cch + 150 μ*M* RHC 80267	53.7 ± 3.2*	52.4 ± 3.7

Modulation of Cch-activated cytosolic PLA_2 and DAG lipase activities by inhibitors. Results for PLA_2 and DAG lipase assays are from six experiments and expressed as means ± SE. Enzymatic assays of cytosolic PLA_2 and DAG lipase were conducted as described in the Methods section. DAG lipase activity was measured by using 1-stearyl-2-[^{14}C] arachidonyl-sn-glycerol as substrate and cytosolic protein extracted from acini as enzyme source. The reaction was carried out in 50 m*M* HEPES buffer, pH 7.4, at 37°C for 30 min. PLA_2 activity was measured by using L-α-1-palmitoyl-2-[^{14}C-arachidonyl] phosphatidylcholine (^{14}C-PAPC) as substrate and cytosolic protein extracted from acini as enzyme source. The reaction was carried out in a 30 m*M* Tris-HCl buffer, pH 8.8, at 37°C for 15 min. For all experiments where inhibitors were used, acini were pretreated with the inhibitors for 15 min. For each experiment, each value was measured in triplicate. Cch, carbamylcholine. *$p < 0.05$ vs their respective control.

Uptake of [^{3}H]-AA into Pancreatic Acini

Uptake of [^{3}H]-AA into pancreatic acini was linear for 120 min and the incorporation of radioactivity into pancreatic phospholipids was also linear for 90 min. Uptake of [^{3}H]-AA into the acini and its incorporation into phospholipids were 0.78 ± 0.06%, 1.02 ± 0.07% of total radioactivity into the cells and 47. 2 ± 1.9%, 53.4 ± 3.8%, of total radioactivity into phospholipids, respectively, after 90 and 120 min of incubation. In light of these initial data, all subsequent studies involved a 90 min labeling period with [^{3}H]-AA. Intracellular accumulation of free [^{3}H]-AA into pancreatic acini and its extracellular release by Cch were significantly improved by 10 μ*M* ETYA, the cyclo-oxygenase and lipoxygenase inhibitor (data not shown). From this observation, this inhibitor was systematically used in all upcoming studies.

Time-Course and Cch-Concentration Dependency on Intracellular Accumulation and Extracellular Release of [^{3}H]-AA

Time-course of 5 μ*M* CCh stimulated AA release was evaluated for up to 60 min. As shown in Fig. 1A, Cch significantly increased intracellular accumulation of [^{3}H]-AA above basal values as early as 5 min for up to 60 min. Similarly, Cch also significantly increased above basal values within 5 min and for at least 60 min, the extracellular release of [^{3}H]-AA into the incubation medium (Fig. 1B). As shown in Fig. 1C, the intracellular accumulation of [^{3}H]-AA into pancreatic acini was Cch-concentration dependent presenting a bell-shaped curve. A significant increase above basal occurred at 1 μ*M*, a maximal increase at 5 μ*M* followed by an inhibition at higher Cch concentrations. The effect of increasing concentrations of Cch on [^{3}H]-AA extracellular release is depicted in Fig. 1D. Similarly, Cch significantly stimulated extracellular AA release at concentrations ranging from 1 μ*M* to 10 μ*M*, with 5 μ*M* being the maximal effective concentration.

Potential Implication of PLA_2 in Cch-Induced [^{3}H]-AA Accumulation and Release

The origin of AA release in the pancreatic acinar cells may be through the sequential action of PLC, PKC, and DAG lipase on phosphatidylinositol (Dixon and Hokin, 1984) or from the breakdown of phosphatidylinositol, phosphatidylethanolamine, or phosphatidylcholine via PLA_2 (Pandol et al., 1991). In this study, two known inhibitors of PLA_2, mepacrine and aristolochic acid (Wallach and Brown, 1981; Spangel et al., 1991) were used to evaluate the potential implication of PLA_2. As shown in Fig. 2A,B,

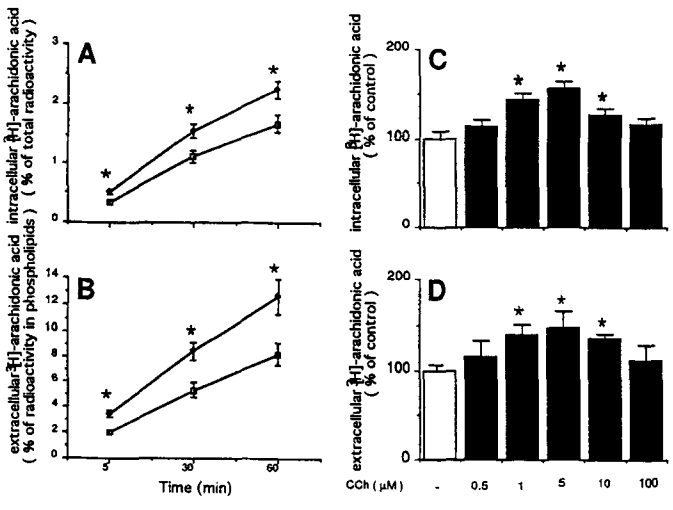

Fig. 1. Time-course **(A, B)** and Cch-dose response curve on intracellular accumulation **(C)** and extracellular release **(D)** of [^{3}H]-AA. After a 15 min pretreatment with 10 μ*M* ETYA, pancreatic acini prelabeled with [^{3}H]-AA for 90 min were incubated for different time periods with 5 μ*M* Cch and for 15 min with increasing concentrations of Cch. Intracellular and extracellular [^{3}H]-AA were measured as described in the Methods section. In these experiments, results represent means ± SE of 5 different experiments. *Significantly different from their respective basal value at $p < 0.05$. ◆–◆, Cch-treated group □, control group.

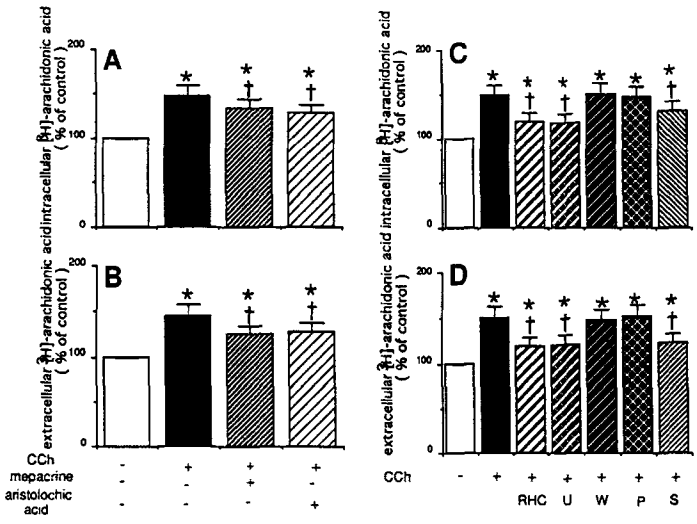

Fig. 2. Effects of PLA_2 inhibitors **(A,B)** and inhibitors of DAG lipase, PLC, PLD, PPH, and PKC **(C,D)** on Cch-stimulated intracellular accumulation and extracellular release of [^{3}H]-AA. Pancreatic acini prelabeled with [^{3}H]-AA for 90 min were treated with 150 μ*M* mepacrine or 200 μ*M* aristolochic acid (A,B), and also treated with 150 μ*M* RHC 80267 (RHC), 10 μ*M* U73122 (U), 50 n*M* wortmannin (W), 200 μ*M* propranolol (P), or 100 n*M* staurosporine (S) for 15 min prior to and during a 15 min stimulation with 5 μ*M* Cch. Intracellular and extracellular [^{3}H]-AA were measured as described in the Methods section. In these experiments, results represent means ± SE of 10 different experiments. *Significantly different from their respective basal value at $p < 0.05$. †Significantly different from Cch-stimulated group at $p < 0.05$.

mepacrine partially inhibited Cch-induced intracellular accumulation and extracellular release of [^{3}H]-AA in pancreatic acini. Similarly to mepacrine, aristolochic acid also suppressed Cch-induced AA liberation (Fig. 2A,B). These data suggest that PLA_2 activation by Cch partially caused the intracellular accumulation of AA into pancreatic acini as well as its extracellular release.

Potential Implication of PLC, PLD, PKC, and DAG Lipase in Cch-Induced [^{3}H]-AA Accumulation and Release

The implication of DAG lipase was first investigated with the selective DAG lipase inhibitor RHC 80267 (Konrad et al., 1994). As indicated in Fig. 2C, 150 μ*M* RHC 80267 partially but significantly inhibited Cch-induced [^{3}H]-AA accumulation into pancreatic acini. The DAG lipase inhibitor also reduced the increment in extracellular AA release caused by Cch (Fig. 2D). The observation that 10 μ*M* U73122, a selective inhibitor of PLC, also inhibited AA release with a comparable efficacy as RHC 80267 suggests that PLC activation by Cch is in line with DAG lipase involvement (Fig. 2C,D). 100 n*M* staurosporine, a known PKC inhibitor, also significantly reduced Cch-induced [^{3}H]-AA accumulation and release (Fig. 2C,D). The selectivity of staurosporine as an inhibitor of pancreatic PKC was further evaluated. Indeed, as shown in Fig. 3A,B, staurosporine effectively blocked the increases in intracellular accumulation and extracellular release of [^{3}H]-AA caused by the PKC activator 4β-phorbol 12 myristate 13 acetate (PMA). The observation that wortmannin, a PLD inhibitor, and propranolol, a PPH inhibitor, did not inhibit the effect of Cch on [^{3}H]-AA accumulation and release (Fig. 2C,D) and that Cch failed to activate PLD activity since PEt production, a specific product of PLD, was 0.18 ± 0.02% and 0.19 ± 0.02% in control and Cch-stimulated groups, respectively, eliminate the PLD pathway. We then set out to further confirm PLC-PKC-DAG lipase and PLA_2 pathways by assessing whether simultaneous addition of a DAG lipase and PLA_2 inhibitors could completely inhibit Cch effect on AA release. As expected, when RHC 80267, the DAG lipase inhibitor, was used in combination with either mepacrine or aristolochic acid, two PLA_2 inhibitors, Cch stimulation on AA liberation was completely inhibited (Fig. 4A,B).

Discussion

Extensive studies have been conducted on the effects of Cch on the hydrolysis of phosphatidylinositol 4,5-bisphosphate and on its action on calcium mobilization. Other studies were also performed on AA release in response to cerulein (Dixon and Hokin, 1984) and CCK-octapeptide (Pandol et al., 1991) in pancreatic acini and on Cch-induced AA release from rat brain cortex membrane (Strosznajder and Samochocki, 1991) and from pancreatic islets (Konrad et al., 1992). As more and more attention is directed toward AA as an important second messenger, mediatory role of AA in the stimulus-secretory coupling has been suggested

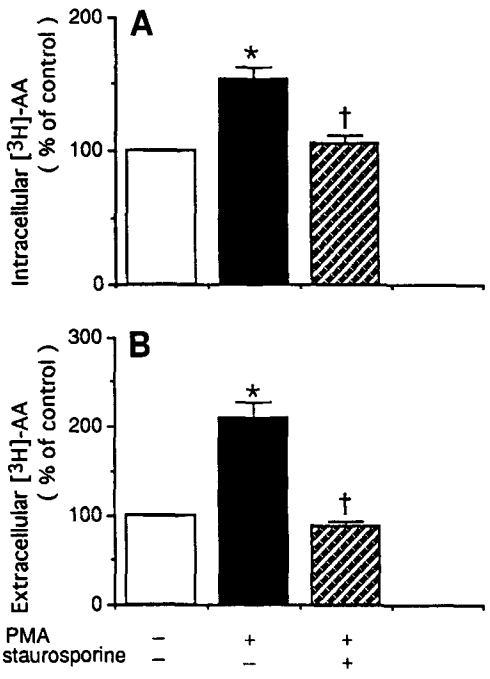

Fig. 3. Effects of the PKC inhibitor staurosporine on PMA-stimulated intracellular accumulation and extracellular release of [^{3}H]-AA. Pancreatic acini prelabeled with [^{3}H]-AA for 90 min were treated with 100 n*M* staurosporine for 15 min prior to and during a 15 min stimulation with 100 n*M* PMA **(A,B)**. Intracellular and extracellular [^{3}H]-AA were measured as described in the Methods section. In these experiments, results represent means ± SE of 5 different experiments. *Significantly different from basal at $p < 0.05$. †Significantly different from PMA-stimulated group at $p < 0.05$.

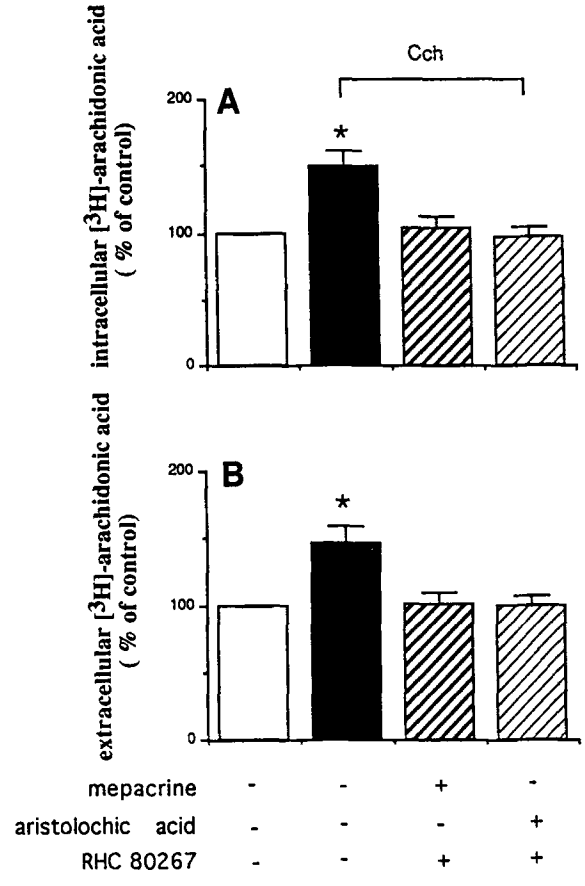

Fig. 4. Inhibition of the effects of Cch on [^{3}H]-AA intracellular accumulation and extracellular release by RHC 80267 given together with either mepacrine or aristolochic acid. Pancreatic acini prelabeled with [^{3}H]-AA for 90 min were stimulated with 5 μ*M* Cch in the presence or not of 150 μ*M* RHC 80267, 150 μ*M* mepacrine, or 200 μ*M* aristolochic acid for 15 min prior to and during a 15 min stimulation. Intracellular and extracellular [^{3}H]-AA were measured as described in the Methods section. In these experiments, results represent means ± SE of 5 different experiments. *Significantly different from basal at $p < 0.05$.

(Dixon and Hokin, 1984; Pandol et al., 1991; Tsunoda and Owyang, 1994). However, an action of Cch on AA release has yet to be reported in the exocrine pancreas. The purpose of this study was to investigate the effects of Cch on AA liberation and furthermore to characterize the intracellular signal transduction pathways that regulate AA release in rat pancreatic acini. There are two major pathways that can generate AA. One involves PLA_2 hydrolysis of membrane phospholipids, whereas the other source of agonist-induced accumulation of AA is from DAG, a product of PLC activation (Dixon and Hokin, 1984). A third pathway may involve PLD catalyzing the hydrolysis of the terminal diester bond of phosphatidylcholine and possibly of other glycerophosphatides with the formation of phosphatidic acid and choline (Cook and Wakelam, 1991). Phosphatidic acid can then serve as a substrate for DAG biosynthesis through the action of PPH (Witter and Kanfer, 1985).

The present study demonstrates for the first time that Cch activates DAG lipase and PLA_2 enzyme activities significantly and stimulates intracellular accumulation and extracellular release of AA in rat pancreatic acini. Cch increased significantly intracellular accumulation and extracellular release of AA in a concentration- and time-dependent manner. Our data confirm a previous demonstration of AA release on stimulation by Cch in rat brain cortex membrane (Strosznajder and Samochocki, 1991) and in pancreatic islets (Konrad et al., 1992). The physiological importance of AA liberation outside the acinar cells still remains to be investigated. Several roles have been postulated for AA. Exogenous application of AA can cause inhibition of inositol incorporation into phosphoinositides in rat pancreatic acinar cells (Chaudhry et al., 1987), elicit Ca^{2+} release from intracellular stores in pancreatic islets (Wolf et al., 1986), increase the number of available GTP-binding proteins and consequently lead to cell activation (Abramson et al., 1991), activate mitogen-activated protein kinase in vascular smooth muscles (Rao et al., 1994), or activate PKC in synergy with DAG (Shinomura et al., 1991). Finally, AA added to pancreatic acini also caused a concentration-dependent increase in amylase release, an effect reproduced by PLA_2 (Pandol et al., 1991).

AA release in response to PLA_2 activation is one of the major pathways involved as previously reported

(Strosznajder and Samochocki, 1991; Currie et al., 1992; Virdee et al., 1994). Our present finding that Cch-stimulated intracellular accumulation and extracellular release of AA was partially inhibited by two PLA_2 inhibitors, mepacrine, and aristolochic acid, supports our observation that Cch activates PLA_2 directly from an enzymatic assay and, therefore, strongly suggests that activation of PLA_2 occurs in response to Cch.

Since AA release is only partially inhibited by mepacrine and aristolochic acid, other intracellular pathways must participate in the Cch response to AA release. The coupled PLD-PPH pathway also able to generate DAG, a substrate for DAG lipase (Exton, 1990), does not *seem* to be involved since propranolol, a known inhibitor of PPH (Witter and Kanfer, 1985), did not suppress Cch-induced generation of AA, ruling out a role for PLD in AA release. Failure of this pathway was further supported by the lack of effect of Wortmannin, a PLD inhibitor (Bonser et al., 1991), to inhibit Cch-induced AA release and also from the convincing observation that Cch was unable to activate PLD, confirming our previous observation (Rivard et al., 1994).

With the absence of the coupled PLD-PPH reactions in AA release, the remaining potential pathway could be a PLC-catalyzed breakdown of phospholipids to form DAG that activates PKC, which in turn activates DAG lipase that then deacylates DAG. Such a pathway has been previously demonstrated by Bell et al. (1979) and Konrad et al. (1994) in human platelets and isolated pancreatic islets, respectively. In Konrad studies on isolated pancreatic islets, it was estimated that combined glucose and Cch-stimulated PLC-DAG lipase pathway activation accounts for approximately two thirds of AA accumulation and secretagogue-induced insulin secretion. In the exocrine pancreas, our estimation of Cch-induced AA accumulation and release (this study) and Cch-stimulated amylase release from pancreatic acini (Hou et al., 1996) is about 50%, PLA_2 activation being responsible for the other 50%. The observation that RHC 80267, a DAG lipase inhibitor, partially inhibited Cch-induced [^{3}H]-AA intracellular accumulation and extracellular release confirms the DAG lipase involvement. These observations were further supported by the evidence that DAG lipase activity increased greatly in response to Cch stimulation and RHC 80267 exhibited inhibitory action on DAG lipase on enzymatic assay. The next question arising from the above observation regards the mechanism by which Cch causes DAG lipase activation and the source of DAG production. Therefore, to ascertain that PLC-DAG lipase activation is the existing pathway, we used the selective PLC inhibitor U73122, capable of a complete inhibition of CCK-OP-stimulated phosphatidylinositol bisphosphate hydrolysis in pancreatic acini (Yule and Williams, 1992). Indeed, the observations that [^{3}H]-AA intracellular accumulation and extracellular release were equally inhibited by U73122 indicate that the activation of PLC by Cch is a prerequisite for DAG lipase increased activity. Incubating pancreatic acini with U73122 also blocked the activation of DAG lipase by Cch.

A previous study has indicated that intracellular AA release can be significantly increased in response to PKC activation by the phorbol ester PMA (Pandol et al., 1991). Our data certainly confirm that initial observation which was further emphasized by the finding that the PKC inhibitor staurosporine totally inhibited PMA-stimulated intracellular AA accumulation and its extracellular release. The demonstration that staurosporine, a known PKC inhibitor, also reduced significantly Cch-induced [^{3}H]-AA intracellular accumulation and extracellular release strongly suggests that activation of DAG lipase is associated with a previous activation of PKC. PKC activation thus, plays an important and obligatory role in the process of sequential action of PLC and DAG lipase.

The simultaneous activation of both PLA_2 and PLC with the subsequent activation of PKC and DAG lipase is thus, considered to be responsible for the Cch effect on AA release. The activation of these two pathways do not quite agree with the single PLA_2 pathway responsible for Cch-mediated AA release from rat brain cortex membrane (Strosznajder and Samochocki, 1991). On the other hand, our data are consistent with those reported by Konrad et al. (1992), demonstrating that both PLC-DAG lipase and PLA_2 were involved in Cch-induced AA accumulation in pancreatic islets. To further confirm these dual pathways as responsible for Cch-induced AA release, we assumed that inhibitors of the PLA_2 and PLC, PKC, and DAG lipase pathways should partially inhibit the effect of Cch on AA release when used separately, with a total inhibition when combined. Our assumptions were indeed totally confirmed since the Cch actions on AA release were partially inhibited by PLA_2 and PLC, PKC, and DAG lipase inhibitors used alone (Fig. 2A–D) and totally inhibited by combination of the PLA_2 and DAG lipase inhibitors (Fig. 4).

AA was reported to participate in the regulation of insulin release from endocrine pancreas (Konrad et al., 1992; Turk et al., 1985). AA-induced Ca^{2+} release was also demonstrated in both endocrine and exocrine pancreas (Tsunoda and Owyang, 1994; Wolf et al., 1986). Following previous reports that AA might be involved in amylase secretion (Dixon and Hokin, 1984; Pandol et al., 1991), a recent study has described that AA is involved in the enzyme secretory process in the exocrine pancreas (Tsunoda and Owyang, 1994). Thus, the possibility exists that AA could be an additional mediator of amylase release. However, although Cch is a well-known potent pancreatic secretagogue and extensive studies have been conducted on its action on the hydrolysis of phosphatidylinositol 4,5-bisphosphate and on the action of inositol trisphosphate on Ca^{2+} mobilization, an effect of Cch on AA release has not yet been reported in the exocrine pancreas. It is, therefore, of great physiological significance to demonstrate a Cch effect on AA release and to further elucidate the intracellular signaling pathways involved.

In conclusion, we demonstrated that Cch activated DAG lipase and PLA_2 enzyme activities and stimulated intracellular accumulation and extracellular release of AA, and elucidated the underlying intracellular signaling pathways for the first time in rat pancreatic acini. Our data do not support any implication of PLD as a prerequisite for the generation of DAG leading to AA liberation. However, our findings indicate that dual pathways, including a sequential action of PLC-PKC-DAG lipase and a PLA_2 action, are responsible for the Cch-stimulated AA liberation.

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