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Activation of Phospholipase C by Cholecystokinin Receptor Subtypes with Different G-protein-Coupling Specificities in Hormone-Secreting Pancreatic Cell Lines

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ABSTRACT. Phospholipase C (PLC) activity was investigated by stimulation of membrane preparations obtained from insulin (β-TC3)-, somatostatin (Rin 1027-B2)-, and glucagon (INR1-G9)-producing pancreatic cell lines using the non-hydrolyzable GTP analogue GTPγS alone, the C-terminal octapeptide cholecystokinin (CCK-8), or gastrin. All compounds caused a significant 2- to 4.4-fold stimulation of PLC activity in the different cell lines, which was diminished by the non-hydrolyzable GDP analogue GDPβS. CCK receptor subtypes were characterized by radioligand binding experiments. High-affinity binding sites for tritiated CCK_A receptor antagonist L-364,718 (K_d = 0.24 nM) and tritiated CCK_B receptor antagonist L-365,260 (K_d = 0.13 nM) were only present in Rin 1027-B2 cells. High-affinity binding sites for both ligands were not found in β-TC3 or INR1-G9 cells. Competition binding experiments with non-labeled CCK receptor antagonists CR 1505 (CCK_A receptor-selective) and CR 2945 (CCK_B receptor-selective), as well as microphysiometry experiments, resulted in the same receptor distribution. Reverse transcriptase–polymerase chain reaction confirmed the CCK receptor distribution pattern for Rin 1027-B2 cells, but in addition showed the existence of CCK_B receptors in β-TC3 cells. Immunoblocking experiments with C-terminal antibodies against different G-protein α-subunits demonstrated inhibition of CCK-stimulated PLC activity in β-TC3 cells by $G_{q/11}\alpha$ antiserum (70%), in Rin 1027-B2 cells by $G_{q/11}\alpha$ antiserum (70%) and G_i-3α antiserum (23%), and in INR1-G9 cells by $G_{q/11}\alpha$ antiserum (60%) and $G_o\alpha$ antiserum (45%). We conclude that CCK receptor subtypes with different G-protein-coupling specificities to PLC are present in the different hormone-secreting cells of the endocrine pancreas. BIOCHEM PHARMACOL **60**;6:865–875, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cholecystokinin receptors; signal transduction; G-proteins; phospholipase C; pancreatic cells

CCK-8§ is known to stimulate insulin secretion *in vivo* [1, 2] and *in vitro* [3, 4] by binding specifically to CCK_A receptors in the pantcreas [5] and has been shown to stimulate phosphoinositide metabolism yielding IP_3 and 1,2-diacylglycerol (DAG) [6]. IP_3 releases Ca^{2+} from intracellular stores while DAG activates protein kinase C (PKC) [7]. It has also been reported that CCK-8 is able to promote the release of glucagon and somatostatin from rat pancreatic islets [3]. Thus, CCK may not only serve as an incretin, but also modify the paracnne effects of hormones in the pancreatic islet.

Pharmacological studies have suggested the existence of two major CCK receptor subtypes in the rat pancreas, the CCK_A and CCK_B/gastrin receptors (for review see [8]). High-affinity CCK receptors have been found in the exocrine pancreas [9] and have been suggested to be present as well in the endocrine pancreas, i.e. islets of Langerhans [10]. Putative CCK receptors have recently been cloned [8, 11] and shown to belong, structurally and functionally, to the superfamily of G-protein-coupled receptors. The involvement of the α-subunits of the pertussis toxin-insensitive heterotrimeric G-proteins of the G_q family [12, 13] in PLC stimulation has been demonstrated for a number of G-protein-coupled receptors [14, 15]. Little is known about the receptor/effector specificity of CCK receptors to PLC in the different endocrine cells located in pancreatic islets.

The purpose of the present study was to gain further insight into the molecular distribution and regulatory function of diverse CCK receptor subtypes in the different cell types located in the endocrine pancreas. We used cell membrane preparations of insulin (β-TC3)-, somatostatin (Rin 1027-B2)-, and glucagon (INR1-G9)- secreting cell lines to investigate the occurrence of different CCK recep-

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§ *Abbreviations:* CCK, cholecystokinin, CCK-8, C-terminal octapeptide of CCK; DAG, 1,2-diacylglycerol; PLC, phospholipase C; PKC, protein kinase C; RT–PCR, reverse transcriptase–polymerase chain reaction; [^{3}H]PIP_2,, phosphatidyl inositol 4,-5-bisphosphate [inositol-2-^{3}H(N)]-PIP_2; ECAR, extracellular acidification rate;; IP_3, inositol trisphosphate; and PVDF, polyvinylidene fluoride.

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tor subtypes and the respective G-proteins involved in the activation of PLC by CCK-8. To our knowledge, investigations regarding CCK receptor distribution and characterization in the different cell types located in the endocrine pancreas had not yet been undertaken. For this purpose, different methods were utilized including saturation and competition radioligand binding experiments and RT–PCR following Southern blot analysis. Receptor/G-protein coupling was investigated using specific antisera directed towards C-terminal sequences of G-protein α-subunits [13, 16, 17], and metabolic activity upon CCK-8 stimulation was measured by microphysiometry.

MATERIALS AND METHODS

Materials

GTPγS and GDPβS were purchased from Boehringer Mannheim and CCK-8, gastrin, and pertussis toxin were obtained from Sigma. PVDF–Immobilon-P membranes were from Millipore, Biotrans nylon membranes were from ICN, and [^{3}H]PIP_2 (5–10 Ci/mmol), [^{3}H]L-364,718 (60–87 Ci/mmol), and [^{3}H]L-365,260 (70–100 Ci/mmol) were purchased from DuPont NEN. Protogel was from National Diagnostics and the enhanced chemiluminescence (ECL) Western blotting system was from Amersham Life Science. CR 1505 (loxiglumide) and CR 2945 were generous gifts from Dr. M. D'Amato.

Cell Culture

β-TC3 [18], Rin 1027-B2 [19], and INR1-G9 [20] cells were maintained in RPMI-1640 medium (11 mM glucose) supplemented with 10% fetal bovine serum, penicillin (75 μg/mL), and streptomycin (50 μg/mL). For the maintenance of β-TC3 cells, the medium was supplemented with 2 mM L-glutamine. Pertussis toxin pretreatment of cells was performed in the presence of 25 ng/mL of pertussis toxin in the culture medium 24 hr prior to cell harvest. Cells were trypsinized and subcloned weekly. The medium was changed twice weekly and 24 hr prior to an experiment. The insulin secretory capacity of the β-TC3 cells in response to 15 mM glucose and 0.5 mM carbachol was monitored regularly by radioimmunoassay (data not shown). The insulin-secreting ability of various β-TC cell lines has been reported to decrease with higher numbers of passage [21]. Therefore, β-TC3 cells of 45–50 passages were used throughout this study.

Preparation of Subcellular Particulate Fractions

The preparation of subcellular particulate fractions of cells was performed as described previously [22], with minor changes. After removal of the medium, the cells were scraped into ice-cold 150 mM NaCl, 10 mM Tris–HCl, pH 7.5 and pelleted (700 × *g*, 10 min, 4°). The cell pellet was washed once with the same buffer, resuspended in 20 volumes of 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, and homogenized on ice using a syringe by aspirating back and forth through a 25-gauge needle for up to six times. The homogenate was then centrifuged at 27,000 × *g* for 30 min at 4°. Finally, the pellet was resuspended in 10 volumes of Tris–EDTA buffer containing 0.1% BSA. This constituted the crude membrane fraction and contained about 3 mg membrane protein per mL. Protein concentration was estimated using the Bradford protein assay (Bio-Rad).

Phospholipase C Assay

This method was adapted from previously published reports [17, 23]. Aliquots (20 μL) of diluted crude membrane suspensions (20–30 μg protein) in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA were mixed with 10 μL incubation mixture containing 100 mM Tris–HCl, pH.6.5, 400 μM GTP, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 μL of CCK-8 (10 μM) or GTPγS (10 μM) and/or GTPβS (4 mM) in 1.6-mL microfuge tubes on ice. A [^{3}H]PIP_2 stock solution in 2% cholate (42,000 cpm/10 μL) was added to each tube, and incubation was carried out at 37° for 5 min. The reaction was stopped by adding in succession 150 μL $CHCL_3$:CH_3OH:HCl (1:2:0.02), 50 μL $CHCl_3$, and 50 μL KCl (2 M). After vortexing and phase separation at 5000 × *g* in a microfuge, 100-μL aliquots of the aqueous layers were counted in a liquid scintillation counter. It is hereby noted that the GTP-dependent PIP_2 hydrolysis results solely in IP_3 formation. However, smaller amounts of inositol diphosphate (IP_2) and inositol monophosphate (IP) can be present in the aqueous phase, due to endogenous phosphatase activity in membranes that acts on IP_3 [23]. In the controls, approximately 2% of the radioactivity was retained in the aqueous phase. The enzyme activity was linear with time up to 30 min and protein concentrations up to 60 μg protein (data not shown).

Radioligand Binding Assays

For radioligand binding assays [24], the cells were grown to subconfluency, rinsed three times for 5 min with cold PBS, and harvested in PBS using a rubber policeman. The cell suspension was centrifuged for 5 min at 1000 × *g*, and the cells were resuspended in cold 50 mM phosphate buffer pH 7.4 containing phenylmethylsulfonyl flouride 1 (0.1 mg/mL), leupeptin (0.5 μg/mL), and DNase (1 μg/mL) and left on ice for 5 min. The cell suspension was then homogenized at 4° using 2 ultrasonic bursts of 10 sec each with a 1-min interval. The suspension was centrifuged for 20 min at 15,000 rpm (Sorvall SS34 rotor) at 4°. The resulting pellet, defined as crude membrane fraction, was resuspended in 50 mM phosphate buffer pH 7.4 containing bacitracin (0.2 μg/mL). Aliquots of the suspensions (30–50 μg protein) were incubated for 90 min at 37° with increasing concentrations of the tritiated non-peptide antagonists L-364,718 (CCK_A antagonist) or L-365,260 (CCK_B antagonist). Nonspecific binding, determined using 1 μM unlabeled CCK-8, was found to be about 40% of total binding and was

subtracted. For competition binding experiments, aliquots were incubated with approximately 1 nM of either [^{3}H]L-364,718 or [^{3}H]L-365,260 and increasing concentrations of CR 1505 (non-labeled CCK_A antagonist) or CR 2945 (non-labeled CCK_B antagonist [25]). Displacement of the L-364,718 with CR 2945 or L-365,260 with CR 1505 resulted in a minor decrease in bound radioactivity of 10% at most at the highest concentrations of antagonists used (>10 μM). In both types of radioligand binding experiments, bound label was separated from unbound radioactivity by filtration using the Skatron micro-cell harvester equipment (Analis). Calculations were performed according to standard procedures [26]. In a separate series of experiments, intact cells in monolayer were washed three times with PBS and subsequently incubated with increasing concentrations of either [^{3}H]L-364,718 or [^{3}H]L-365,260 in PBS. After 60 min at 25°, cells were washed three times with PBS followed by one wash with ice-cold glycine buffer (50 mM glycine, 125 mM NaCl, pH 3.0 with HCl) for 5 min. Finally, cells were solubilized overnight in 1 M NaOH. All washes were analyzed by liquid scintillation counting.

Western Blotting and Immunostaining

SDS–(PAGE) was performed as described [27]. Cell membrane samples were prepared for electrophoresis by suspending in sample buffer and heating to 100° for 5 min. Proteins were electrophoretically transferred [28] from gel to PVDF–Immobilon-P membranes with constant current (100 mA) for about 14 hr in a Bio-Rad transblot apparatus. After transfer, the filter was incubated for 2 hr at room temperature with PBS containing 5% non-fat dry milk and 0.1% Tween 20, and subsequently incubated for 6–24 hr at room temperature in PBS containing 0.1% Tween 20 and the respective G-protein α-subunit antisera. The filter was then washed extensively with PBS containing 0.1% Tween 20, and proteins were visualized by enhanced chemiluminescence according to the manufacturer's protocol.

G-protein α-Subunit Antisera

Rabbit antisera against synthetic peptides corresponding to the predicted C-terminal amino acid sequence of different G-protein α-subunits (denominated QL, AS, GO, EC, and RM) were a generous gift from Dr. A. M. Spiegel, NIH Bethesda, MD, U.S.A. [14, 16, 29]. The $G_i1\alpha/G_i$-2α antiserum (AS) detects both the G_i-1α and G_i-2α subunits, and the $G_o\alpha$ (GO) antiserum displays some reciprocal cross-reactivity to G_i-3α. The $G_q\alpha$ antiserum (QL) detects both $G_q\alpha$ and $G_{11}\alpha$. The $G_s\alpha$ (RM) and G_i-3α (EC) antisera display no cross-reactivity. The antisera were used at a final dilution of 1/200–1/400 for Western blot analysis.

Pretreatment of Cell Membranes with G-protein α-Subunit Antisera

Crude membrane fractions were prepared as described above, and the antiserum treatment was carried out as previously described [17]. Aliquots of diluted membrane suspensions (0.3 mg/mL) were incubated with C-terminal antisera and non-immune rabbit antiserum at a final serum concentration of 3.5 μg/mL for 1–2 hr at 4° prior to enzyme assays.

Northern Blot Analysis

mRNA was isolated directly from cell samples with the QuickPrep mRNA Purification Kit (Pharmacia Biotech) according to the manufacturer's protocol. Five micrograms of mRNA was separated on 1.2% agarose gels in 18% formaldehyde and 3-[*N*-morpholino]propane-sulphonic acid (MOPS) buffer and blotted onto Biotrans nylon filters (ICN) in Tris-acetate–EDTA buffer as described previously [30]. ^{32}P-Labeled cDNA probes of CCK_A and CCK_B receptors [8, 11, 31] and β-actin [32] were made from gel-purified cDNA fragments by random priming. Plasmids containing cDNA clones for CCK_A and CCK_B receptors were kindly provided by Dr. Stephen Wank (NIH Bethesda, MD, U.S.A.). RNA detection was carried out according to Maniatis *et al.* [30], with slight modifications that have been described previously [22].

Amplification of CCK Receptor Precursor cDNAs by PCR

cDNA synthesis and amplification of precursor cDNA was performed with Ready-to-Go RT–PCR beads (Pharmacia Biotech) according to the manufacturer's protocol. The PCR was carried out with primers that correspond to the nucleotide sequence of the rat CCK_A receptor precursor cDNA [11, 33] (sense primer, nucleotides 317–337, AGTCTGCACTGCAGATTCTCC; antisense primer, nucleotides 791–811, TAGCGTCACTTGGCAACAGG). For amplification of the rat CCK_B receptor precursor cDNA [31, 33], the following primers were used: nucleotides 1276–1297, CACTTGCTGAGCTACGTCTCTG and nucleotides 1848–1870, GTCACTTCTGCACTAGGCTATGG. Primers that correspond to the rat β_2-microglobulin precursor cDNA [33] (nucleotides 284–303, ACCGAGACCGATGTATATGC and nucleotides 373–392, TGATTCAGATCTCCATAGAG) were used as an internal standard. Cycles were carried out at 98° for 3 min, 55° for 30 sec, and 72° for 1 min (1 cycle), and 95° for 30 sec, 55° for 3 min, and 72° for 3 min (35 cycles). The resulting PCR products were separated on 1.8% agarose gels and bands were visualized by staining with ethidium bromide.

TABLE 1. CCK-8-, gastrin-, and GTPγS -modulated PLC activity in β-TC3, Rin 1027-B2, and InR1-G9 cell membranes

Cells	Phospholipase C activity (10^3 cpm/mg membrane protein/min)			
	Basal	CCK-8	Gastrin	GTPγS
β-TC3	9.36 ± 0.12	19.32 ± 0.31*	18.66 ± 0.29*	26.81 ± 0.35*
INR1-G9	10.63 ± 0.16	46.51 ± 0.23*	27.49 ± 0.20*	54.04 ± 0.34*
Rin 1027-B2	44.42 ± 0.23	123.40 ± 0.65*	117.80 ± 0.53*	141.98 ± 0.81*

Crude membrane fractions (20 μg) of (β-TC3, Rin 1027-B2, and INR1-G9 cells were assayed for PLC activity in the presence or absence of 2.5 μM CCK-8, gastrin, or GTPγS. Enzyme activities are expressed as 10^3 cpm of IP_3 formed per mg protein per min. Values are given as means ± SEM of four independent experiments.
*Indicates $P < 0.05$ when compared to the appropriate control.

Southern Blot Analysis of PCR-Amplified Products

PCR-amplified products were separated on 1.8% agarose gels and blotted onto Biotrans nylon membranes (ICN). ^{32}P-Labeled cDNA probes of CCK_A or CCK_B receptors were hybridized for 16 hr at 37° and washed subsequently with 2 × saline citrate solution (SSC), 0.1% SDS for 5 min at room temperature and 0.1 × SSC, 0.1% SDS for 15 min at 65° prior to autoradiography.

Microphysiometry

Cytosensor experiments were performed according to the manufacturers' protocol [34]. The cell cycle was set to 3 min and the pump was halted during the last 25 sec during which the acidification rate was calculated; pump speed was 100 μL/min. Agonists were CCK-8 and gastrin; only the results with CCK-8 were suitable for calculation because of the weak response upon gastrin stimulation (data not shown). Antagonists (0.1 μM final concentration) were given 30 min before agonist stimulation. Introduction of the antagonists had no effect on the basal acidification rate. For the calculation of the ECAR, the average of four basal ECAR values before stimulation was normalized to 100% and all other values were expressed as percentages thereof.

Statistical Evaluation

For statistical analysis the Friedman two-way analysis of variance was used [35]. When significance was reached, each experimental condition was compared with the appropriate control including correction for multiple comparisons. Statistical significance was accepted when $P < 0.05$.

RESULTS

Phospholipase C Activity in Different Hormone-Producing Pancreatic Cell Lines

Basal-, CCK-8-, gastrin-, and GTPγS-dependent PLC activity was measured in membrane preparations of insulin (β-TC3)-, somatostatin (Rin 1027-B2)-, and glucagon (INR1-G9)- secreting cell lines. The cells were stimulated with CCK-8, gastrin, or GTPγS. The results are summarized in Table 1. Basal PLC activity was significantly higher in Rin 1027-B2 cells (about 4-fold) than in β-TC3 and INR1-G9 cells, where the basal activity was comparable. CCK-8 and gastrin were able to stimulate PLC activity in all cell lines, although to a different extent. The activities ranged from 2-fold in β-TC3 cells and 2.8-fold in Rin 1027-B2 cells for CCK-8 and gastrin, and 4.4-fold for CCK-8 and 2.6-fold for gastrin in INR1-G9 cells when compared to their corresponding basal activities. The PLC activity was linear up to 10 μM CCK-8 or gastrin (data not shown). GTPγS-mediated stimulation of PLC activity was slightly higher than for CCK-8: 2.9-fold for β-TC3 cells, 5.2-fold for INR1-G9 cells, and 3.2-fold for Rin 1027-B2 cells. The relative increase in PLC activity upon stimulation was close to equal for these cell types. In addition, the PLC activity was partially pertussis toxin-insensitive in INR1-G9 cells (data not shown). GDPβS (1 mM) reduced basal PLC activity by approximately 20%, and CCK-8- and GTPγS-stimulated PLC activity was diminished in all three cell lines (data not shown).

Western Blot Analysis of the Relative Distribution of G-protein α-Subunits in Membrane Preparations from Different Hormone-Producing Pancreatic Cell Lines

As shown in Fig. 1, $G_{q/11}\alpha$ protein (approximately 42 kDa) was found in various quantities in pancreatic cell types: there appeared to be equal amounts of $G_{q/11}\alpha$ protein in β-TC3 and INR1-G9 cells, while somewhat less was found in Rin 1027-B2 cells. The distribution pattern of G_i-1/$_i$-2α protein (approximately 40 kDa) was the same in the different pancreatic cell lines as found for $G_{q/11}\alpha$. $G_o\alpha$ protein (approximately 40 kDa) was more abundant in Rin 1027-B2 cells than in β-TC3 and INR1-G9 cells. G_i-3α protein (approximately 41 kDa) appeared to be more abundant in INR1-G9 cells then in β-TC3 and Rin 1027-B2 cells. $G_s\alpha$ protein (approximately 46 kDa [upper arrow] and 42 kDa [lower arrow]) was found in various quantities in the pancreatic cell types: there appeared to be considerably more $G_s\alpha$ protein of the high-molecular form (46 kDa) in INR1-G9 cells than a β-TC3 and Rin 1027-B2 cells. The low-molecular-weight form of the $G_s\alpha$ protein (42 kDa) was found to be present in equal amounts in β-TC3 and INR1-G9 cells, but was nearly absent in Rin 1027-B2 cells.

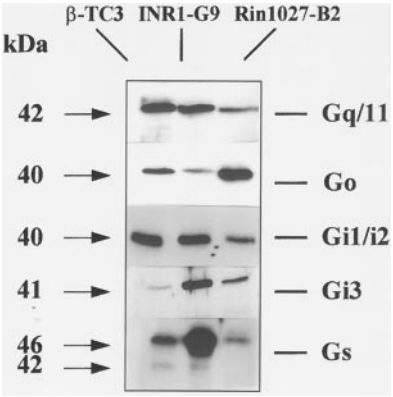

FIG. 1. Immunoblot analysis of G-protein α-subunit distribution in β-TC3, Rin 1027-B2, and INR1-G9 cell membranes. Crude membrane fractions (20 μg per lane) were separated by 10% SDS–PAGE and transferred to PVDF membranes as described in Materials and Methods. The presence of G-protein α-subunits was detected with antisera against C-terminal sequences of $G_{q/11}\alpha$ (approx. 42 kDa), G_i-1/$_i$-2α (approx. 40 kDa), $G_o\alpha$ (approx. 40 kDa), G_i-3α (approx. 41 kDa), and $G_s\alpha$ (approx. 46 kDa and 42 kDa), and visualized by enhanced chemiluminescence. The distribution pattern was confirmed by three different experiments.

Immunoblocking of CCK-Stimulated Phospholipase C Activity in Different Hormone-Producing Pancreatic Cell Lines

The hormone receptor G-protein-coupling specificity for cholecystokinin was investigated using C-terminal antisera of $G_{q/11}\alpha$, G_o, G_i-3, G_i-1/$_i$-2α, and $G_s\alpha$ subunits and rabbit non-immune serum. Membrane fractions of the different pancreatic cell lines were pretreated with the antisera prior to the PLC assay. The activation of PLC by CCK-8 was measured in untreated (control) and pretreated membranes. The results of the experiments are summarized in Fig. 2. The $G_{q/11}\alpha$ antiserum blocked the CCK-mediated phospholipase C activation in all cell lines tested, although to a different extent. The highest inhibition was observed for β-TC3 cells (approximately 70%). In INR1-G9 and Rin 1027-B2 cells, $G_{q/11}\alpha$ antiserum blocked CCK-8-stimulated PLC activity by approximately 43% and 55%, respectively. In INR1-G9 cells, CCK-8-elicited PLC activation was also inhibited by $G_o\alpha$ antiserum by approximately 30%; in the other cell lines, the $G_o\alpha$ antiserum was essentially without effect. The G_i-3α antiserum blocked the CCK-8-elicited PLC activation only in Rin 1027-B2 cells, by approximately 23%. Antisera against G_i-1/$_i$-2α, $G_s\alpha$, and rabbit non-immune serum (NRS) had no significant effect on the ability of CCK-8 to modulate phospholipase C activity in any cell line tested.

Identification of CCK Receptor Subtypes in Different Hormone-Producing Pancreatic Cell Lines

Results on the identification of CCK receptor subtypes by saturation radioligand binding are shown in Fig. 3. In the crude membrane preparation of Rin 1027-B2 cells, high-affinity binding sites for [^{3}H]L-364,718 (K_d = 0.24 nM, Fig. 3A) and [^{3}H]L-365,260 (K_d = 0.13 nM, Fig. 3B) were found. In the β-TC3 cells, non-specific binding for both ligands was very high (between 50 and 80% of total binding), and reproducible Scatchard plots could not be obtained. In INR1-G9 cells, no high-affinity binding sites for either ligand could be detected (data not shown). The results of the competition binding experiments are shown in Fig. 4. The competition between [^{3}H]L-364,718 and CR 1505 (both CCK_A antagonists) and [^{3}H]L-365,260 and CR 2945 (both CCK_B antagonists) demonstrated efficient competition in Rin 1027-B2 cells. Competition between

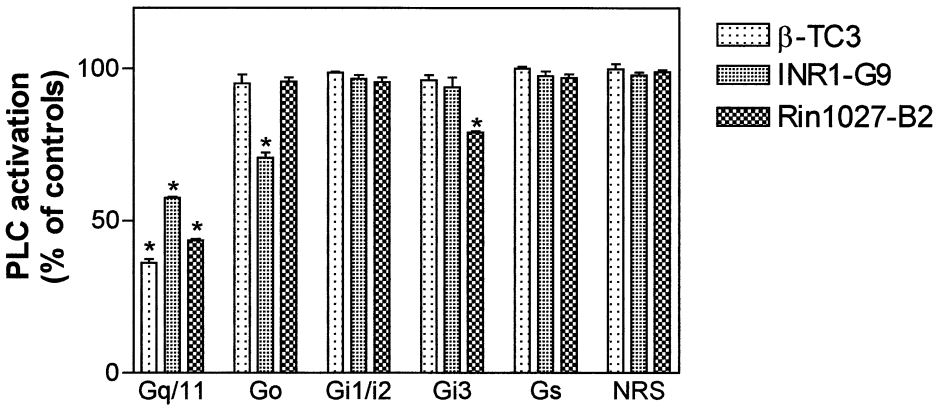

FIG. 2. The effect of Gα C-terminal antiserum pretreatment on CCK-8-modulated phospholipase C activity. Aliquots of β-TC3, INR1-G9, and Rin 1027-B2 cell membrane suspensions were incubated with Gα C-terminal antisera ($G_{q/11}$, G_i-1/$_i$-2, G_i-3, G_o, and G_s) and non-immune rabbit antiserum (NRS) as indicated, at a final concentration of 3.5 μg/mL. Subsequently, the membranes were assayed for CCK-8-modulated PLC activity. The PLC activity is expressed as percentage of controls (actual CCK-8-stimulated PLC activation of non-treated cell membranes), which were set to 100%. The figure shows the means ± SD of four independent experiments, each performed in triplicate. Statistical analysis was performed as described in Materials and Methods. (*) denotes $P < 0.05$ when compared to controls. For basal- and CCK-8- modulated PLC values (10^3 cpm IP_3/mg protein/min), see Table 1.

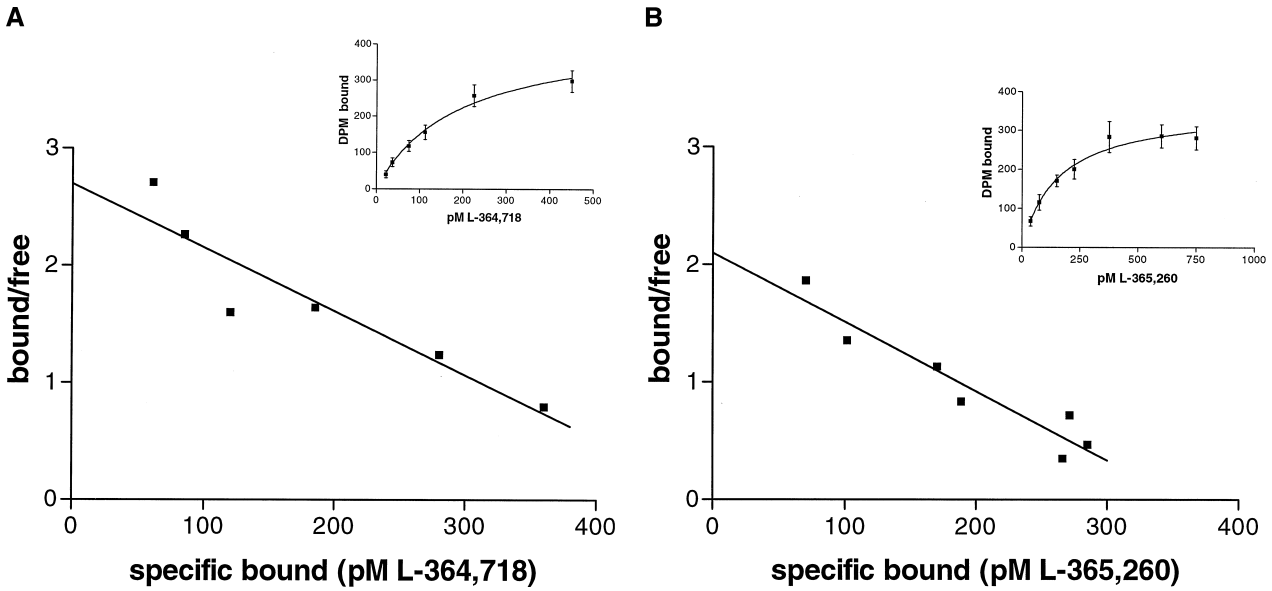

FIG. 3. Identification of CCK receptor subtypes. Representative example of $n = 3–8$ of the Scatchard plot and corresponding saturation radioligand binding (insert) obtained with [^{3}H]L-364,718 (CCK_A-selective ligand; Fig. 3A) and [^{3}H]L-365,260 (CCK_B-selective ligand; Fig. 3B) to a crude membrane preparation of Rin 1027-B2 cells. Incubation conditions are depicted in Materials and Methods and unlabeled CCK-8 (1 μM) was used to determine non-specific binding.

L-364,718 and CR 2945 or between L-365,260 and CR 1505 was at most 20% of maximal binding (data not shown). When intact cells in monolayer were incubated according to the protocol described in Materials and Methods, no saturable binding was found independent of the ligand used (data not shown).

Expression of CCK_A and CCK_B Precursor Genes in Different Hormone-Producing Pancreatic Cell Lines

The expression of CCK_A and CCK_B receptor precursor genes in β-TC3, INR1-G9, and Rin 1027-B2 cells was studied by RT–PCR and following Southern blot analysis. The results are shown in Fig. 5. Amplified cDNA was obtained from mRNA of Rin 1027-B2 cells, that corresponds to a PCR fragment of the expected molecular size of 495 bp (Fig.5A) from the CCK_A receptor precursor mRNA. These fragments were not obtained in β-TC3 and INR1-G9 cells. Southern blot analysis resulted in a single hybridized band of approximately 0.5 kb (Fig. 5B). From mRNA of β-TC3 and Rin 1027-B2 cells, a DNA fragment of the expected size of 595 bp was obtained from the CCK_B receptor precursor mRNA (Fig. 5C). Here, Southern blot analysis revealed a single hybridizing fragment of 0.6 kb (Fig. 5D). The β_2-microglobulin precursor mRNA was used as an inner standard for the RT–PCR reaction and appeared in all cell lines as a single DNA fragment with an expected size of 109 bp (data not shown).

Measurements of CCK-Stimulated Metabolic Activity in Different Hormone-Producing Pancreatic Cell Lines

Figure 6 depicts the results of the physiometry experiments. CCK-8 caused a small but significant dose-dependent ECAR (absolute value is between 10% and 15% above basal ECAR) in Rin 1027-B2 cells only. From the curve, the EC_{50} value is calculated to be 4.6 ± 0.6 nM ($n = 4$). The response was blocked by CR 1505 (CCK_A antagonist; Fig. 6A) and by CR 2945 (CCK_B antagonist; Fig. 6B) at a concentration of 0.1 μM. Higher concentrations of CR 2945 caused a strong and irreversible decline in basal ECAR that was probably due to cell death (data not shown). This phenomenon was not investigated further. In β-TC3 and INR1-G9 cells, no reproducible change in ECAR upon CCK stimulation was obtained (data not shown).

DISCUSSION

We here report on the activation of PLC via different CCK receptor subtypes and G-protein coupling in different hormone-secreting cells of the endocrine pancreas. As model systems, we have used tumor-derived cell lines that produce the pancreatic hormones insulin (β-TC3 cells), glucagon (INR1-G9 cells), and somatostatin (Rin 1027-B2 cells). All cell lines displayed basal PLC activity that was further activated by the sulfated fragment of cholecystokinin (CCK-8) or gastrin. The basal, CCK-, and gastrin-stimulated PLC activity measured in Rin 1027-B2 cells differed significantly from that in INR1-G9 and β-TC3 cells and was possibly a side effect of transformation. CCK-stimulated PLC activity increased in the following order: β-TC3 cells < INR1-G9 cells < Rin 1027-B2 cells. Maximum activation of G-proteins by the non-hydrolyzable analogue GTPγS could reflect the net effect of regulatory signals on this enzyme system.

Earlier reports have shown that CCK stimulates the

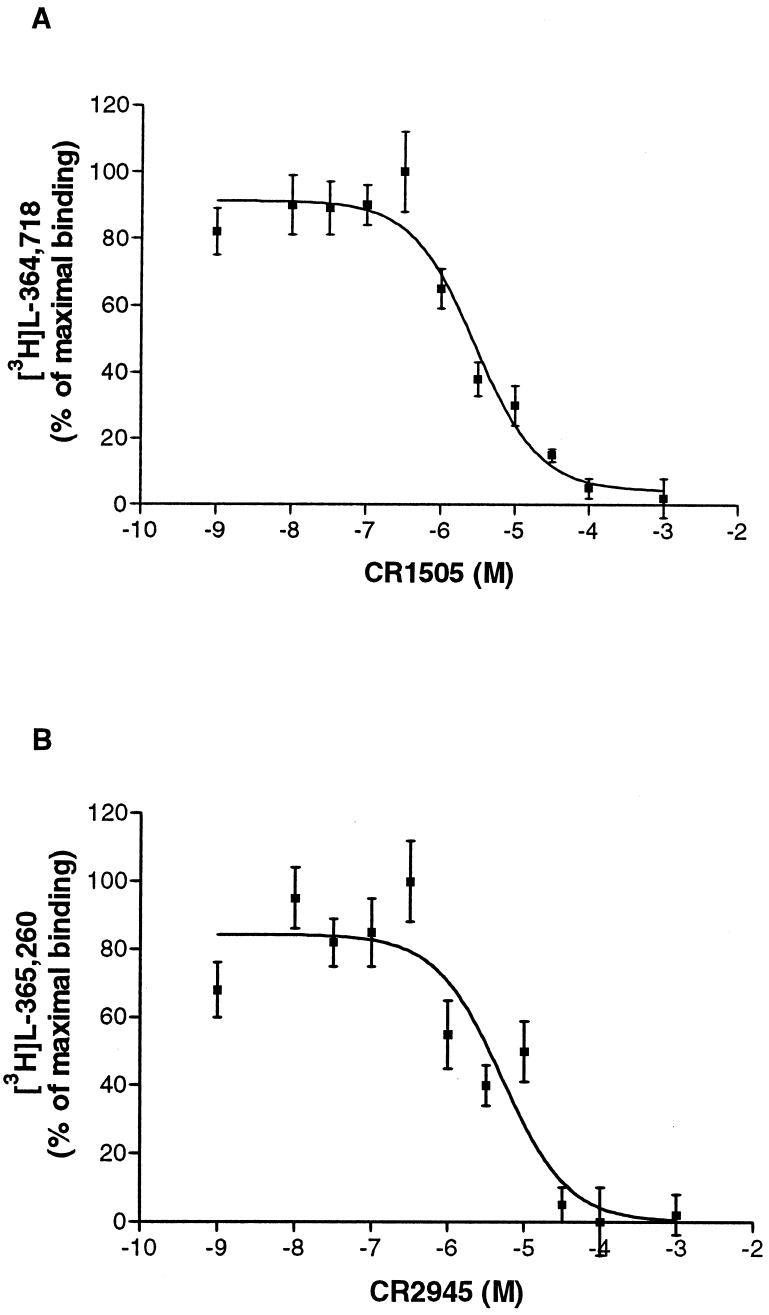

FIG. 4. Competition radioligand binding experiment using [^{3}H]L364718 and CR 1505 (both CCK_A antagonists; Fig. 4A) or [^{3}H]L365260 and CR 2945 (both CCK_B antagonists; Fig. 4B) in Rin 1027-B2 cells. Radioligand binding without competitor was set to 100%.

release of insulin and glucagon from the endocrine pancreas both *in vivo* and *in vitro*, probably via CCK_A receptors [2, 4, 36]. In addition, pharmacological studies have led to the common view that different CCK_A receptors are located in the insulin-producing β-cells of the endocrine pancreas [10]. However, CCK receptor distribution in the different hormone-producing cells located in the endocrine pancreas has not yet been investigated. Using three different experimental approaches (radioligand binding, RT–PCR, and microphysiometry), we attempted to identify the subtype of CCK receptors that could be responsible for induction of hormone release in the cell lines. In Rin 1027-B2 cells, our results clearly show that both CCK_A and CCK_B receptors were present. Radioligand saturation binding experiments with the two selective commercially available radioligands ([^{3}H]L-364,718 for CCK_A receptor and [^{3}H]L-365,260 for CCK_B receptor) yielded acceptable Scatchard plots. For [^{3}H]L364718, the presence of high-affinity binding sites (low nanomolar range) was demonstrated, with the affinities comparable to those described previously [37, 38]. Surprisingly, the affinity for [^{3}H]L-365,260 was in the same low nM range, which is in contrast to what is generally accepted for the CCK_B ligand. To our knowledge, this ligand has never been used in this cell line; hence, comparison to the affinity obtained in COS cells transfected with the CCK_B receptor gene (K_i of about 3 nM [39]

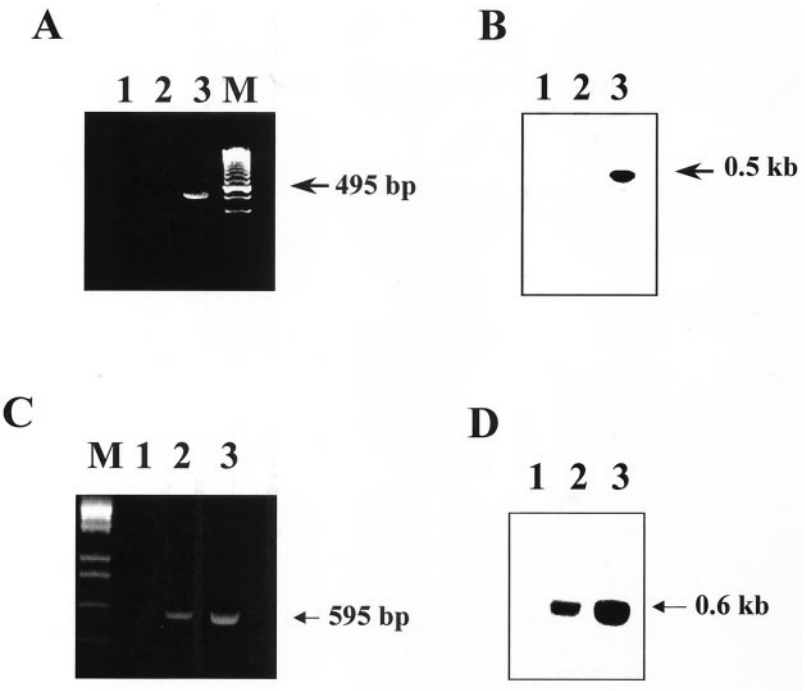

FIG. 5. Expression of CCK_A and CCK_B receptor precursor genes. The coding regions of the CCK_A receptor precursor (495 bp) (A) and CCK_B receptor precursor cDNA (595 bp) (C) were amplified by RT–PCR. The amplified products were fractionated on a 1.8% agarose gel and subsequently stained with ethidium bromide. Lanes: M, 100-bp DNA ladder molecular weight standard (GIBCO BRL); 1, INR1-G9 cells; 2, β-TC3 cells; 3, Rin 1027-B2 cells. Autoradiograms of the RT–PCR-amplified cDNA fragments of the CCK_A receptor (B) and CCK_B receptor (D) by Southern blot hybridization with radiolabeled full-length CCK_A and CCK_B receptor probe. The Southern blots displayed single bands of 0.5 kb (CCK_A receptor) and 0.6 kb (CCK_B receptor), respectively. Lanes: M, 100-bp DNA ladder molecular weight standard (GIBCO BRL) in (A) and 1 kb plus DNA ladder molecular weight standard (GIBCO BRL) in (C); 1, INR1-G9 cells; 2, β-TC3 cells; 3, Rin 1027-B2 cells. The experiments were repeated twice with similar results.

and IC_{50} of 2–6 nM [40]) should be made with caution. It bears mentioning that our initial approach, i.e. the use of iodinated CCK-8 as ligand, failed to yield reproducible results and therefore was abandoned in favor of the tritiated ligands. In β-TC3 cells, ligand binding experiments were less successful. In this cell line, non-specific binding was very high (up to 80 % of total binding at the K_d value), which renders Scatchard plots useless. Therefore, no conclusion as to the presence of CCK receptors from ligand binding experiments was possible for this cell line. For the INR1-G9 cells, no saturation or specific binding was obtained within the concentration of ligand used. Hence, we conclude that the radioligand binding assay failed to demonstrate the presence of CCK receptors with high affinity for both ligands.

The second approach involved Northern blot analysis and RT–PCR followed by Southern blot analysis. The attempt to study the expression of different CCK receptor subtypes by Northern blot analysis failed (data not shown). This may have been due to a generally low expression of CCK receptor type mRNA, as has been reported previously [41]. Therefore, we utilized the RT–PCR technique to determine CCK receptor subtype distribution in the different cell lines. With this technique, we were able to detect CCK_A receptors only in Rin 1027-B2 cells, whereas CCK_B receptors could be detected in both Rin 1027-B2 and β-TC3 cells. The results obtained are in agreement with those yielded by receptor binding assays. Again, neither of the CCK receptor subtypes could be detected in INR1-G9 cells. For Rin 1027-B2 cells, both groups of obtained data were compatible, indicating the presence of CCK_A and CCK_B receptors. For the β-TC3 cells, the situation is less clear. The expression levels of CCK receptors can be very low and, in combination with less favorable conditions for thermodynamic interactions, ligand binding studies can be disappointing. In this respect, we believe that the RT–PCR results lead to the conclusion that CCK_B receptors are present in β-TC3 cells.

The third approach involved microphysiometry. This method for measuring receptor-mediated enhanced metabolic acidity leading to enhancement of the ECAR has been shown to be very valuable for the study of signal transduction systems of G-protein-coupled receptors. The method was recently used for HeLa cells transfected with the CCK_B receptor gene [39]. Our experiments demonstrate that this method can also be used for a non-transfected cell line with a low expression level of CCK receptors. Our results again indicate the presence of (physiological active) CCK receptors in Rin 1027-B2 cells, while the use of antagonists (loxiglumide and CR 2945) clearly demonstrate the presence of CCK_A and CCK_B receptors. In β-TC3 and INR1-G9 cells, no effect was seen. It shown previously that in certain cell types, the ECAR is very low even upon strong stimulatory conditions. We conclude that in these cell lines either the transduction between receptor stimulation and ECAR is absent or no CCK receptors are present. In view of the above-mentioned results, it is obvious that this method is not suitable for β-TC3 and INR1-G9 cells. The results obtained, based on three different experimental approaches, show that both CCK_A and CCK_B receptors are present in Rin 1027-B2 cells, while RT–PCR analysis indicates the presence of CCK_B receptors in β-TC3 cells only. In INR1-G9 cells, it is tempting to speculate that a CCK receptor with unknown specificity is present, although the present methodology does not allow its detection. Examples for yet unknown potentially novel

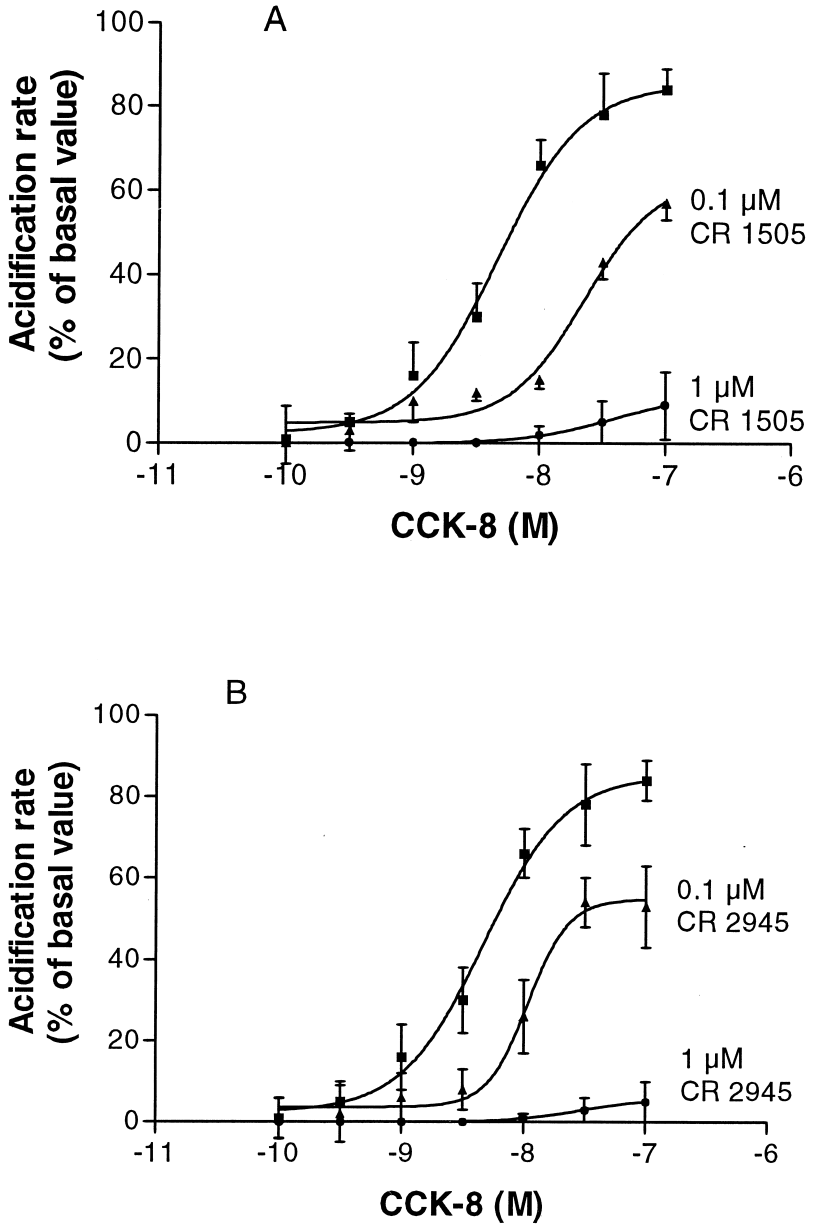

FIG. 6. Microphysiometry experiments. Inhibition of the CCK-8 response (■) on the ECAR by 0.1 μM CR 1505 (▲) and 1 μM CR1505 (●) (CCK_A antagonist, Fig. 6A.) and by 0.1 μM CR 2945 (▲) and 1 μM CR2995 (●) (CCK_B antagonist, Fig. 6B.) in Rin 1027-B2 cells. Data are calculated as described in Materials and Methods. Curves show the average value of four independent experiments with the average value ± SEM indicated.

CCK receptor subtypes have also been suggested by others [42].

For the elucidation of the specificity of receptor/effector coupling, G-protein-specific antisera raised against G-protein α-subunits were used to examine which G-proteins are involved in mediating the activation of PLC by CCK-8. The C-terminus of G-proteins is involved in G-protein/receptor interaction, but not in effector coupling [13, 16]. The specific antisera block receptor/G-protein interaction without causing global inhibition of G-protein function. Immunoblocking experiments have shown that $G_{q/11}\alpha$ alone is responsible for the activation of PLC by CCK in pancreatic acinar cells [43]. Our results demonstrate that PLC activation in endocrine pancreatic cells involves both pertussis toxin-insensitive and pertussis toxin-sensitive G-proteins. In β-TC3 cells, PLC is activated solely by $G_{q/11}\alpha$. It should be noted that it is not possible to distinguish between $G_q\alpha$ and $G_{11}\alpha$, since the antiserum is directed against the C-terminal part of $G_q\alpha$ and $G_{11}\alpha$, which is identical [44]. However, the CCK receptors in INR1-G9 cells and Rin 1027-B2 cells seem to be promiscuous in coupling to different G-protein species. In addition to $G_{q/11}\alpha$-triggered PLC activation, pertussis toxin-sensitive signaling pathways could be identified that include $G_o\alpha$ in INR1-G9 cells and G_i-3α in Rin 1027-B2 cells. The activation of PLC triggered through pertussis toxin-sensitive G-protein α-subunits is possibly due to "cross-talk" between two different second messenger systems [45]. This could explain the participation of G_i-3α in the activation of PLC in Rin 1027-B2 cells. We have found two receptor subtypes in Rin 1027-B2 cells that can be stimulated with the non-selective agonist CCK-8. The selectivity of ago-

nists for CCK_A and CCK_B receptors is, however, poor, and a conclusion as to the selective stimulation is therefore not possible. Activation of PLC through $G_o\alpha$ has been reported earlier [46]. In addition to $G_q\alpha$ and $G_{11}\alpha$, α-subunits of G_{14}, G_{15}, and G_{16} proteins are able to activate PLC-β [47]. $G_{14}\alpha$ is expressed in a variety of cells and could therefore also be involved in the agonist-induced activation of PLC-β in the pancreatic cell lines. However, anti-$G_{14}\alpha$ antibody was not available during the course of this study. The expression of G_{15} and G_{16} α-subunits appears to be restricted to hematopoetic cells [12], making the possibility that these G-proteins participate in the regulation of PLC-β in the pancreatic cell lines unlikely.

In the present study, we have shown for the first time that distinct hormone-producing cells of the endocrine pancreas contain CCK receptor subtypes with different coupling specificities to GTP-binding proteins in the activation of PLC. Further investigations into the effect of CCK and gastrin on PLC activation and hormone secretion are presently underway in our laboratory. This may clarify the role of cholecystokinin in regulation of hormone production and secretion in the different cell types of the endocrine pancreas.

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