CCK, carbachol, and bombesin activate distinct PLC-\(\beta\) isoenzymes via \(G_{q/11}\) in rat pancreatic acinar membranes

Piiper, Albrecht, Danuta Stryjek-Kaminska, Rolf Klengel, and Stefan Zeuzem. CCK, carbachol, and bombesin activate distinct PLC-\(\beta\) isoenzymes via \(G_{q/11}\) in rat pancreatic acinar membranes. Am. J. Physiol. 272 (Gastrointest. Liver Physiol. 35): G135-G140, 1997.-Four different isoforms of phospholipase C-\(\beta\) (PLC-\(\beta_{1-4}\)) have been discovered, raising the important question of whether a distinct receptor activates a single PLC-\(\beta\) isoenzyme or a subset of PLC isoenzymes. The present study was designed to investigate activation of PLC-\(\beta\) isoenzymes by three different PLC-activating agonists that bind to different receptor entities, i.e., cholecystokinin octapeptide (CCK-8), bombesin, and carbachol in rat pancreatic acinar membranes. PLC activity was measured using exogenous \([^{3}H]\)phosphatidylinositol 4,5-bisphosphate as substrate. Western blot analysis of pancreatic acinar membranes revealed the presence of PLC-\(\beta_{1}, \beta_{2}, \gamma_{1}\), and \(-\delta_{1}\) but not of PLC-\(\beta_{3}, \delta_{1}, \gamma_{2}\), and \(-\delta_{2}\). Preincubation of the membranes with anti-PLC-\(\beta_{1}\) or -\(\beta_{3}\) antibody reduced agonist-induced activation of PLC. The order of sensitivity toward inhibition by anti-PLC-\(\beta_{1}\) antibody was CCK-8 > bombesin > carbachol. An opposite order of sensitivity was found for inhibition of PLC activity by anti-PLC-\(\beta_{3}\) antibody (carbachol > bombesin > CCK-8). Anti-PLC-\(\beta_{2}\), \(\beta_{4}, \gamma_{1}, \gamma_{2}, -\delta_{1}\), and \(-\delta_{2}\) antibodies had no effect. Preincubation of the membrane with an agonist raised against the COOH terminus of \(\alpha\)-subunit of PLC-\(\alpha_{1}\) proteins inhibited PLC activity in response to all three different receptor agonists to a similar extent, whereas anti-\(G_{i1}\), anti-\(G_{i3}\), and anti-\(G_{i4}\) antibodies had no effect. In conclusion, the data of the present study indicate that CCK-8 and carbachol activate PLC-\(\beta_{1}\) and PLC-\(\beta_{3}\), respectively, whereas bombesin activates both PLC-\(\beta_{1}\) and PLC-\(\beta_{3}\). Activation of PLC-\(\beta\) by these receptor agonists is mediated by \(G_{q/11}\).

cholecystokinin; phospholipase C-\(\beta\); signal transduction

MATERIALS AND METHODS

CCK-8, carbachol, bombesin, collagenase type III, phosphatidylinositol 4,5-bisphosphate, phosphatidylethanolamine, soybean trypsin inhibitor, and affinity-purified horseradish peroxidase-hemocyanin-conjugated anti rabbit and anti mouse immuno globulin G (IgG) were obtained from Sigma Chemical (St. Louis, MO). Mixed monoclonal anti-PLC-\(\beta_{1}\) and anti-PLC-\(\gamma_{1}\) antibodies were from Upstate Biotechnology (Lake Placid, NY). Enhanced chemiluminescence (ECL) reagents and films were obtained from Amersham (Braunschweig, Germany). \([^{3}H]\)phosphatidylinositol 4,5-bisphosphate (6 Ci/mmol), polyclonal rabbit G1-\(\alpha_{1}, \alpha_{2}, G_{i1}, G_{i3}, G_{i4}\) and \(G_{i1}\) antibodies were from Du Pont-New England Nuclear (Bad Homburg, Germany). An antibody raised against an internal sequence of \(G_{i1}\) (283–300) was from Calbiochem (La Jolla, CA). Polyclonal anti-PLC-\(\beta_{1}, \beta_{2}, \beta_{3}, \beta_{4}, \gamma_{1}, -\delta_{1}\), and \(-\delta_{2}\) were from Santa Cruz (Santa Cruz, CA).

Preparation of isolated rat pancreatic acini. The preparation of isolated rat pancreatic acini was performed as described recently using a collagenase digestion method (36).

Isolation of membranes. A fraction enriched in plasma membranes was isolated from pancreatic acini essentially as described recently (27). Pancreatic acini were homogenized by 20 strokes using a glass Teflon potter in 10 vol of homogenization medium containing (in mM) 250 sucrose, 0.1 MgSO4, 0.2 phenylmethylsulfonflury fluoride, and 50 N-2-hydroxyethylpiiperazine-N'-2-ethanesulfonic acid, pH 7.0, as well as 5 \(\mu\)g/ml leupeptin, 5 \(\mu\)g/ml aprotinin, and 0.01%...
soybean trypsin inhibitor (wt/vol). The homogenate was centrifuged twice at 150 g for 10 min at 4°C and once at 13,000 g for 10 min at 4°C to remove unbroken cells, debris, nuclei, and zymogen granules. The resulting supernatant, containing mainly plasma membrane and endoplasmic reticulum, was centrifuged at 27,000 g for 25 min at 4°C, resuspended in fresh homogenization medium at a protein concentration of 30 mg/ml, and stored in liquid nitrogen until use. Protein was measured according to the method of Bradford (2), using bovine serum albumin as a standard.

**RESULTS**

**Effect of anti-\(G_{q/11}\alpha\) and anti-\(G_{a}\) antibodies on agonist-induced PLC activity.** Pancreatic acini are known to express the pertussis toxin-sensitive G proteins \(G_{i1}, G_{i2}, G_{i3},\) and \(G_{a}\) (23), as well as the cholera and pertussis toxin-insensitive G protein \(G_{q}\) (26). Pretreatment of isolated pancreatic acini for 4 h with pertussis toxin (20 \(\mu\)g/ml) has no effect on inositol phosphate accumulation in response to the CCK-8 analogue caerulein (21), carbachol, or bombesin (data not shown). Thus G proteins of the cholera toxin- and pertussis toxin-insensitive family \(G_{a}\) rather than \(G_{i}\) and \(G_{q}\) are likely to couple the respective receptors to phosphoinositide hydrolysis. To study the G proteins mediating agonist-induced PLC activation more directly, PLC activity was measured in isolated pancreatic acinar membranes preincubated with antibodies raised against the COOH-terminal part of \(\alpha\)-subunits of G proteins. An antibody raised against the \(\alpha\)-subunit of \(G_{q}\) and \(G_{i1}\) bound to a 42-kDa substrate in pancreatic acinar membranes (Fig. 1A). As shown in Fig. 2, CCK-8 (10 nM), bombesin (100 nM), and carbachol (10 \(\mu\)M) increased basal PLC activity in pancreatic acinar membranes 3.0-, 3.3-, and 4.0-fold, respectively (\(n = 5\)). Preincubation of the membranes with this antibody inhibited PLC activation by all three different receptor agonists with similar potency (CCK-8, 60%; carbachol, 55%; bombesin, 46%; Fig. 2). A similar inhibitory effect of the anti-\(G_{q11}\alpha\) antibody was observed at lower concentrations of these receptor agonists (data not shown).

Half-maximal inhibition of CCK-8-induced PLC activation was observed at an antibody concentration of 10 \(\mu\)g IgG/ml (data not shown). An antibody raised against an internal sequence of \(G_{q11}\alpha\) had no effect on CCK-8-induced PLC activation (data not shown). In contrast, anti-\(G_{a1-2}\) and anti-\(G_{a2}\) antibody, which cross-reacts with \(G_{a}\), had no effect on agonist-stimulated PLC

**Immunoblotting.** Pancreatic acinar membranes (50 \(\mu\)g of protein) were boiled for 4 min in Laemmli buffer (17) and separated on sodium dodecyl sulfate-polyacrylamide gels under reducing conditions. Gel-resolved proteins were electrotransferred to nitrocellulose sheets. The nitrocellulose membrane was first washed for 15 min with tris(hydroxymethyl)aminomethane (Tris)-buffered saline containing 0.05% Tween 20 (wt/vol) (TBST) and was then blocked with 5% skim milk (wt/vol) in TBST for 1 h, followed by washing twice with TBST. The blocked nitrocellulose paper was incubated with the appropriate primary antibody at a dilution of 1:1,000 in blocking buffer. Antigen-antibody complexes were visualized using appropriate horseradish peroxidase-conjugated antibodies and the ECL system.

**Assay of PLC activity.** Phosphatidylinositol 4,5-bisphosphate-specific PLC activity in pancreatic acinar membranes was assayed essentially as previously described (4). In brief, 30 \(\mu\)g of pancreatic acinar membrane protein were incubated for 20 min at 30°C in a final volume of 70 \(\mu\)l containing 28 \(\mu\)M \(\beta\)-hydroxyethylamine (Tris)-buffered saline containing 0.05% Tween 20, 280 \(\mu\)M phosphatidylethanolamine, 50 mM Tris-HCl, pH 7.4, 2.8 mM ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 80 mM KCl, 10 mM LiCl, 10 \(\mu\)M GTP, 10 mM 2,3-bisphosphoglycerate, 1.2 mM sodium desoxycholate, and CaCl\(_2\) to obtain 1 \(\mu\)M free Ca\(^{2+}\). The reaction was terminated by adding 350 \(\mu\)l of CHCl\(_3\)-CH\(_2\)OH-concentrated HCl containing 5 mM EGTA (500:500:3, vol/vol). Radioactivity released into the upper aqueous phase was quantitated by liquid scintillation counting. The data presented are means ± SE of at least three separate experiments with assays performed in triplicate. Statistical analysis was performed using Student’s t-test for paired values.

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**Fig. 1.** Presence of \(G_{q11}\alpha\) (A) and phospholipase C (PLC) isoenzymes (B) in pancreatic acinar membranes. Western blots of pancreatic acinar membranes (50 \(\mu\)g protein/lane) were probed with anti-\(G_{q11}\alpha\) antibody (A) or anti-PLC-\(\beta_1\), \(\beta_2\), \(\beta_3\), \(\beta_4\), \(\gamma_1\), \(\gamma_2\), and \(\gamma_3\) antibodies (B), and immune complexes were visualized by peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence system. Data shown are representative for 3 independent experiments.
AGONIST-INDUCED ACTIVATION OF PLC-β ISOENZYMES

Fig. 2. Effect of anti-Gq11α, anti-Gq11β, and anti-Gq11δ antibodies on CCK-8-, carbachol-, and bombesin-induced activation of PLC. Pancreatic acinar membranes were preincubated with anti-Gq11α, anti-Gq11β, or anti-Gq11δ antibody (50 μg immunoglobulin G (IgG)/ml each) for 2 h at 4°C before assessment of PLC activation in response to CCK-8 (10 nM), carbachol (10 μM), and bombesin (100 nM). Data means ± SE of 3 different experiments. **Significant differences, P < 0.01.

Activation (Fig. 2). Thus these data suggest that activation of PLC-β1 and -β3 by the three different receptor agonists is mediated by Gq11 proteins.

Effect of anti-PLC antibodies on CCK-8-, bombesin-, and carbachol-induced PLC activity. Western blot analyses revealed the presence of PLC-β1, -β3, -γ1, and -δ1 in pancreatic acinar membranes, whereas PLC-β2, -β4, -γ2, and -δ2 were not detectable (Fig. 1B).

Anti-PLC antibodies were tested for their ability to inhibit agonist-induced PLC activation. Polyclonal anti-PLC-β1 antibodies showed differential effects on agonist-induced activation of PLC (Fig. 3). The order of sensitivity of agonist-stimulated PLC activity toward inhibition by anti PLC-β1 antibody was CCK-8 (77% inhibition, P < 0.001) > bombesin (62% inhibition, P < 0.001) > carbachol (21% inhibition, P < 0.05). Half-maximal inhibition of CCK-8-induced PLC activation was observed at an anti-PLC-β1 antibody concentration of 0.3 μg IgG/ml (Fig. 4A). A similar result was obtained when membranes were preincubated with a mixed monoclonal anti-PLC-β1 antibody, confirming that the inhibitory effect of the anti-PLC-β1 antibody was specific.

Preincubation of the membranes with anti-PLC-β3 antibody strongly reduced carbachol (10 μM)-induced PLC activity (64% inhibition, P < 0.001) and partially inhibited bombesin (100 nM)-induced PLC activation (39% inhibition, P < 0.001; Fig. 3). CCK-8 (10 nM)-induced PLC activity was not significantly influenced by the anti-PLC-β3 antibody, indicating that the inhibitory effect of this antibody on carbachol- and bombesin-induced PLC activation was specific. Half-maximal inhibition of carbachol-induced PLC activation was observed at an anti-PLC-β3 antibody concentration of 0.4 μg IgG/ml (Fig. 4B). Anti-PLC-β2, -β4, -γ1, -γ2, -δ1, and -δ2 antibodies had no effect on CCK-, carbachol-, or bombesin-induced PLC activation (Table 1).

Preincubation of the membranes with both anti-PLC-β1 and -β3 antibody (10 μg/ml each) led to an
smooth muscle membranes (22, 23) and that anti-

of PLC is inhibited by an anti-PLC-\(\gamma\) antibody in

the CCK receptor is coupled to PLC-\(\gamma\) is supported by

recent studies showing that CCK-8-induced activation

protein \(\gamma\)-subunit heterotrimer modulate agonist-

factors other than the expression of a particular G

PLC-\(\gamma\) isoenzymes in isolated pancreatic acinar mem-

units were used to investigate differential activation of

bombesin activates both PLC-\(\gamma\) isoenzymes to a similar

activate PLC-\(\gamma\) and PLC-\(\gamma\) respectively, whereas

branes. The data show that CCK-8 and carbachol

receptor), which have been cloned and belong to the

class of heptahelical receptors (9). The contention that

py-subunit has been reported to activate PLC-\(\gamma\) as

appears to be restricted to hematopoetic cells (40),

and pertussis toxin-pretreatment of pancreatic

induced activation of PLC-\(\gamma\) in pancreatic acini. How-

cells (40) and could therefore be involved in agonist-

activation of distinct PLC isoenzymes suggests that other PLC-

coupled receptors also activate a distinct subset of

PLC-\(\gamma\) isoenzymes. The functional implication of differen-
tial PLC isoenzyme activation is unclear. It is inter-
esting to note that acetycholine and CCK evoke differ-
et patterns and spatial organization of Ca\(^{2+}\) oscil-
ations in pancreatic acinar cells (18). An attractive idea is that

activation of distinct PLC isoenzymes induces a dis-
tinct pattern of Ca\(^{2+}\) signaling.

In pancreatic acinar membranes the anti-G_{q/11}\(\alpha\) antibody
inhibited activation of PLC by CCK-8, bombesin, and
carbachol approximately to the same extent. Partial
inhibition is suggestive of other operative pathways.

Anti-G_{q}\(\alpha\) and anti-G_{q}\(\alpha\) antibodies had no effect on

CCK-8-, carbachol- and bombesin-induced PLC activity,

and pertussis toxin-pretreatment of pancreatic

acini did not alter activation of PLC by CCK-8 (21),
carbachol, or bombesin (data not shown). In addition to

G_{q}\(\alpha\) and G_{q}\(\alpha\), \(\alpha\)-subunits of G_{14}, G_{15}, and G_{16} proteins
activate PLC-\(\beta\) (41). G_{q}\(\alpha\) is expressed in a variety of

cells (40) and could therefore be involved in agonist-

induced activation of PLC-\(\beta\), in pancreatic acini. How-

ever, anti-G_{q}\(\alpha\) antibody was not available in the pres-
ent study. The expression of G_{15} and G_{16} proteins
appears to be restricted to hematopoetic cells (40),
excluding the possibility that these G proteins partici-
pate in regulation of PLC-\(\beta\) isoenzymes in pancreatic acini.

Another possibility is that \(\beta\)-\(\gamma\)-subunits of G proteins
partially mediate agonist activation of PLC-\(\beta\)

isoenzymes in pancreatic acinar membranes. G protein
\(\beta\)-\(\gamma\)-subunit has been reported to activate PLC-\(\gamma\), as
well as PLC-\(\beta\) and PLC-\(\gamma\) (35). However, the high
number of G protein \(\beta\) and \(\gamma\)-subtypes makes it
difficult to determine the exact subunit composition of
the G protein heterotrimer(s) coupling an individual

Table 1. Effect of anti-PLC-\(\beta_1\), -\(\beta_2\), -\(\beta_3\), -\(\beta_4\), -\(\gamma_1\), -\(\gamma_2\), -\(\delta_1\), and -\(\delta_2\) on CCK-8-, carbachol-, and bombesin-induced activation of PLC

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Data are means ± SE of 3 independent experiments. Pancreatic acinar membranes were preincubated with anti-phospholipase C (PLC)-\(\beta_1\), -\(\beta_2\), -\(\beta_3\), -\(\beta_4\), -\(\gamma_1\), -\(\gamma_2\), -\(\delta_1\), and -\(\delta_2\) antibodies at a concentration of 10 \(\mu\)g immunoglobulin G/ml or the appropriate amount of normal rabbit serum (control) for 2 h at 4°C before assessment of PLC activation in response to CCK-8 (10 nM), carbachol (10 \(\mu\)M), and bombesin (100 nM).
receptor to a distinct PLC-β isoenzyme by PLC assay and immunoneutralizing antibodies. Partial inhibition of agonist-induced PLC activation may also be due to incomplete immunoneutralization of $G_{q11}$.

Immunoneutralization of G proteins by antibodies raised against the COOH-terminus of G protein α-subunits is an established method to probe for involvement of a G protein in signal transduction (22, 23). These antibodies abolish interaction and thus activation of G proteins by receptors (7). The finding that an antibody raised against the COOH-terminus of α-subunit of Gq/11 protein inhibited activation of PLC in response to all three different receptor agonists, although CCK-8 activates PLC-β1 and carbachol activates PLC-β3, suggests that the coupling of different receptors to different PLC isoenzymes is mediated by the same G protein, i.e., $G_{q11}$. $G_{q1}$ and $G_{q10}$ share indistinguishable properties for activation of PLC-β1 and -β3 in vitro (8). Moreover, multiple combinations of G protein βγ-subunits were uniformly active on PLC-β3 (38). This raises the important question of what confers receptor-effector specificity in situ. A possibility is that specific receptors and distinct PLC isoenzymes are colocalized. In this localized environment, activation of G proteins would lead to activation of a certain PLC-β isoenzyme. Evidence supporting this assumption comes from experiments that demonstrate that PLC is a guanosine triphosphatase (GTPase)-activating protein (GAP) for $G_{q}$ (1). The observations that the induced GTPase activity exceeded the bulk rate of guanine nucleotide exchange and that hydrolysis of only a portion of the $G_{q}$ GTP was stimulated suggest that this GAP activity is due to a subpopulation of receptors and $G_{q}$ proteins, which are in close association, with rapid turnover. Membrane organization of receptors, G proteins, and effectors may be facilitated by interaction with cytoskeletal elements (28).

A further hypothesis is that signaling specificity is determined in part by proteins found in the receptor's microenvironment, which, together with the receptor, G protein, and effector, contribute to the formation of a signal-transduction complex at the cytoplasmic face of the receptor. This hypothesis is consistent with data showing the existence of multimeric G protein complexes and the isolation of receptor or G protein subunits together with some effectors (5, 20, 24, 39).

In conclusion, the results of the present study demonstrate strong selectivity of particular receptors for PLC-β subtypes. The data show that CCK-8 and carbachol activate PLC-β1 and PLC-β3, respectively, whereas bombesin activates PLC-β1 and -β3 to a similar extent. CCK-8, carbachol-, and bombesin-induced activation of PLC-β1 and -β3 depends on $G_{q11}$.

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