Mutation of Asn-391 within the Conserved NPXXY Motif of the Cholecystokinin B Receptor Abolishes $G_q$ Protein Activation without Affecting Its Association with the Receptor

Among the most conserved regions in the G-protein-coupled receptors is the (N/D)PX$_{2–3}$Y motif of the seventh transmembrane domain ($X$ represents any amino acid). The mutation of the Asn/Asp residue of this motif in different G-protein-coupled receptors was shown to affect the activation of either adenyl cyclase or phospholipase C. We have mutated the Asn residue (Asn-391) of the NPXXY motif in the CCKBR to Ala and determined the effects of the mutation on binding, signaling, and G-proteins coupling after expression of the mutated receptor in COS cells. The mutated receptor displayed similar expression levels and high affinity CCK binding compared with the wild type CCKBR. However, unlike the wild type CCKBR, the mutated receptor was completely unable to mediate activation of either phospholipase C and protein kinase C-dependent and -independent mitogen-activated protein kinase pathways, indicating an essential role of Asn-391 in CCKBR signaling. Coimmunoprecipitation experiments allowed us to show that the inactive mutant retains an intact capacity to form stable complexes with $G_\alpha$ subunits in response to CCK. These results indicate that the formation of high affinity CCK-receptor-$G_q$ protein complexes is not sufficient to activate $G_q$ and suggest that Asn-391 is specifically involved in $G_q$ proteins activation.

Cholecystokinin receptors belong to the superfamily of G-protein-coupled receptors that are characterized by seven transmembrane $\alpha$-helical domains (TM) connected by extracellular and intracellular loops. Cholecystokinin binds with high affinity and exerts multiple physiological functions through two pharmacological subtypes, CCKAR and CCKBR (1–3). It is well described that CCKBR stimulates phospho-

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Activation of Gq Protein by Cholecystokinin B Receptor

**Experimental Procedures**

**Materials**—The sulfated carboxyl-terminal nonapeptide of CCK (Thr28, Nle31)-CCK25-33 referred in the article as CCK-9 was synthesized as described previously (16). (Thr28, Nle31)-CCK25-33 was conjugated with Bolton-Hunter reagent (BH), purified, and radiiodinated as described previously (17) and referred to as 125I-BH-CCK-9. Gqα cDNA subcloned in pCIS vector (pCIS-Gqα) was provided by Dr. M. Simon (18). Anti-hemagglutinin (HA) mouse monoclonal antibodies (12CA5 clone) and rat monoclonal antibodies (3F10 clone) were from Roche Molecular Biochemicals. Rabbit polyclonal antibodies against Gqα/G12α were obtained by Dr. M. N. Dufour. Protein A-Sepharose-CL4B, aprotonin, leupeptin, phenylmethylsulfonyl fluoride, and bovine serum albumin 70S were purchased from Sigma. The pCMV-β-galactosidase reporter plasmid was kindly provided by Dr. H. Paris.

**Construction of Mutant Receptor cDNAs**—Mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (QuickChange™ Site-directed Mutagenesis Kit, Stratagene, France), using the rat CCKBR cDNA subcloned in pCDL-SRα (pCDL-SRα-WT-CCKBR) as template. Mutations were confirmed by DNA sequencing using an automated sequencer (Applied Biosystems).

**Transfection of Wild Type and Mutant Receptor cDNAs into Mammalian Cells**—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum. Usually 2 m of the transfection medium were used for the transfection experiments. After transfection, the transfected cells were incubated over-night in Dulbecco's modified Eagle’s medium with 2 mM of 125I-BH-CCK-9 (125I-BH-CCK-9) as template. Mutations were confirmed by DNA sequencing using an automated sequencer (Applied Biosystems).

**Measurement of Gqα Protein Expression by Cholecystokinin B Receptor**—The mutant N391A-CCKBR and wild type N134-I (Thr28, Nle31)-CCK25-33 referred in the article as CCK-9 was always less than 10% of total binding. Binding data were measured using an ECAI Bertold luminometer. Luciferase activity was expressed as relative light units and normalized for β-galactosidase activity. β-Galactosidase activity was measured by the luminescent light derived from 90 μl of each sample incubated in 200 μl of 1 ml/μg O-nitrophenyl-β-D-galactopyranoside and used to correct transfection efficiency among the different treatment groups.

**Immunoprecipitation of Receptor-G Protein Complexes**—As described previously (20), a peptide consisting of PDQDVDPYA, from bovine thymus (17) and referred to as125I-BH-CCK-9. Gqα was always less than 10% of total binding. Binding data were measured using an ECAI Bertold luminometer. Luciferase activity was expressed as relative light units and normalized for β-galactosidase activity. β-Galactosidase activity was measured by the luminescent light derived from 90 μl of each sample incubated in 200 μl of 1 ml/μg O-nitrophenyl-β-D-galactopyranoside and used to correct transfection efficiency among the different treatment groups.

**Western Blotting Analysis**—Proteins (whole cell lysates or immunoprecipitates), prepared as described above, were separated by SDS-polyacrylamide gel electrophoresis on 5/15% linear gradient and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). After transfer, the membranes were saturated with saline buffer (10 mM Tris, 140 mM NaCl, 0.1% Tween, 10% glycerol (pH 7.5)) and then eluted with Laemmli buffer (50 μM Tris, 10% glycerol, 1% β-mercaptoethanol). Membrane proteins were visualized by autoradiography.

**RESULTS**

The N391A-CCKBR Mutant Bound CCKBR Agonists and Antagonists with High Affinity—To determine if the conserved Asn residue of the NFXXY motif (Asn-391) plays a role in binding and activation of the CCKBR receptor, we have mutated this Asn to Ala. The mutant N391A-CCKBR and wild type (WT) CCKBR receptors were transiently expressed in COS-7 cells. We first determined the affinity of N391A-CCKBR for CCK and several selective CCKBR agonists and antagonists by performing competition binding experiments using 125I-BH-CCK-9 as radioligand. Scatchard analysis of CCK-9 competition binding to the WT-CCKBR and N391A-CCKBR mutant revealed that the N391A-CCKBR mutant has an affinity for a maximal binding capacity close to the WT-CCKBR (Kd = 0.21 ± 0.41 nM and Bmax = 1.31 ± 1.18 pmol/106 cells versus Kd = 1.40 ± 0.34 nM and Bmax = 1.28 ± 0.53 pmol/106 cells, respectively).

Moreover, the N391A-CCKBR mutant bound CCK-4 and gastrin-17 agonists with an affinity similar to the WT-CCKBR, whereas it bound the selective CCKBR antagonists, PD135-158 and L365-260, with slightly increased affinities (Table I). These

| Kd (nM) | Agonists | Antagonists |
|--------|----------|-------------|
|        | CCK-4    | G17-I       |
|        | 5.70 (±2.11) | 3.17 (±0.36) |
|        | G17-I    | L365-260    |
|        | 17.6 (±2.34) | 7.16 (±2.95) |

TABLE I

Affinities of different agonists and antagonists for the WT-CCKBR and N391A-CCKBR mutant transiently expressed in COS cells
data indicate that the mutation of Asn-391 to Ala neither affects the high affinity binding of CCKBR agonists and antagonists nor the cell surface receptor expression.

The N391A-CCKBR Mutant Is Unable to Stimulate PLC and MAPK Pathways—We then investigated the effects of this mutation on the main signaling pathways activated by the CCKBR transiently expressed in COS-7 cells. PLC activation mediated by the WT-CCKBR has been well described in COS cells (4, 20, 21). To assess the ability of the wild type and mutant receptors to activate the PLC-signaling cascade, we measured the accumulation of total inositol phosphates (IP). As illustrated in Fig. 1, CCK stimulated total IP production to the WT-CCKBR with an efficacy of 0.94 ± 0.03 nm and a maximal potency of 11-fold over basal. Under similar experimental conditions, no detectable IP accumulation was measured with the N391A-CCKBR mutant even when exposed to 10⁻⁶ M CCK. This result indicates that Asn-391 is essential for activation of the PLC pathway.

We then investigated the ability of WT-CCKBR and N391A-CCKBR mutant to activate the MAPK pathway. Both protein kinase C-dependent and -independent pathways were described to mediate CCKBR-MAPK activation in different cell lines (5, 6). Since no such studies have been performed in COS cells, we first assessed whether the WT-CCKBR expressed in COS-7 cells was able to activate MAPK in response to CCK. We used Elk-1 and luciferase gene reporting systems to measure CCK-induced MAPK activation.Activation of MAPK is known to target transcription factors such as Elk-1 that regulates the activity of the promoter of the early response gene c-fos that was reported to be induced by the CCKBR (26, 27).

As shown in Fig. 2, CCK induced luciferase activity in a concentration-dependent manner with an efficacy of 11.3 ± 0.8 nM. A maximal increase of 18.5-fold in luciferase activity over basal was reached when WT-CCKBR-transfected cells were stimulated with 10⁻⁶ M CCK. This effect was inhibited in a concentration-dependent manner by the highly specific MAPKK1–2 inhibitor PD98059 (28) (Fig. 3). These data indicate that CCK activates in COS cells a cascade of phosphorylation reactions that targets p42 and p44 MAPK. To determine if a PKC-independent pathway might be involved in CCK-induced MAPK activation, we used the PKC-specific inhibitor bisindolylmaleimide GF109203X (29). As shown in Fig. 4A, treatment of the cells with 5 μM GF109203X completely abolished the PMA-stimulated MAPK activity. At a similar concentration, GF109203X inhibited 64% of CCK-induced MAPK activity (Fig. 4B), indicating that CCK-induced MAPK activation is both dependent and independent on PKC. We then determined if the N391A-CCKBR mutant was able to activate MAPK. Similar experiments were performed with COS-7 cells expressing the N391A-CCKBR mutant. No luciferase activity was detectable even at a concentration of 10⁻⁴ M CCK (Fig. 5).

These results indicate that the mutation of Asn-391 to Ala completely inhibits both PKC-dependent and -independent activation of p42 and p44 MAPK.

CCK Binding on N391A-CCKBR Mutant Is Not Affected by GTPγS and AlF₄⁻—To determine if the impairment of mutant receptor activity could be associated to a default in G-protein activation, we measured the effects of the hydrolysis-resistant GTP analogue GTPγS on ¹²⁵I-BH-CCK-9 binding to membrane preparations from COS-7 cells expressing the wild type and mutant receptors. As shown in Fig. 6, treatment with increasing concentrations of GTPγS decreased the high affinity binding of ¹²⁵I-BH-CCK-9 to the WT-CCKBR by about 75%. In contrast, the high affinity binding of ¹²⁵I-BH-CCK-9 to the N391A-CCKBR mutant was not affected by concentrations of GTPγS as high as 10⁻⁴ M. We also determined the effect of AlF₄⁻, which binds to the GoGDP and mimics the gamma phosphate of GTP, on cells expressing the wild type and mutant receptors. In the presence of 30 mM NaF plus 10 μM AlCl₃, concentrations that we previously showed to affect high affinity binding of CCK to the WT-CCKBR (22), the N391A-CCKBR mutant exhibited unchanged high affinity CCK binding, whereas the WT-CCKBR showed a 90% decrease in CCK binding (Fig. 7). It is generally assumed that the high affinity state of agonists binding reflects the formation of a ligand-receptor-G-protein complex, and addition of guanine nucleotides or aluminum fluoride should reduce or eliminate such high affinity binding. The fact that the high affinity CCK binding to the
As described under "Experimental Procedures", COS-7 cells were cotransfected with pCDL-SRα-WT-CCKBR, pFA2-Elk-1, pFR-Luciferase, and the pCMV-β-galactosidase plasmids. Then, cells expressing the WT-CCKBR and the N391A-CCKBR mutant were treated with 5 μM GF109203X for 30 min, exposed to 10 nM PMA for 7 h, and were then assayed for luciferase activity. As described above, transfected cells were treated with 5 μM GF109203X, a concentration that completely abolished PMA-stimulated luciferase activity, and either pCDL-SRα-WT-CCKBR or pCDL-SRα-N391A-CCKBR plasmids. The data represent the mean ± S.E. of three to four separate experiments each performed in duplicate.

### Fig. 5. Stimulation of Elk-1 transcriptional activity by CCK in COS cells expressing the WT-CCKBR and N391A-CCKBR mutant.

As described under "Experimental Procedures," COS-7 cells were cotransfected with pFA2-Elk-1, pFR-Luciferase, pCMV-β-galactosidase, and either pCDL-SRα-WT-CCKBR or pCDL-SRα-N391A-CCKBR plasmids. Then, cells expressing the WT-CCKBR and the N391A-CCKBR were exposed for 7 h to 10⁻⁶ and 10⁻⁴ μM CCK, respectively, and assayed for luciferase activity. Luciferase activity, normalized to β-galactosidase, is expressed as fold induction over basal (untreated cells). The data represent the mean ± S.E. of four separate experiments, each performed in duplicate.

N391A-CCKBR mutant is not affected in presence of GTPγS or ATP might suggest that the mutant retained the capacity to interact with G-proteins but not to activate them. To determine whether the N391A-CCKBR mutant was able to couple to G-proteins when stimulated by CCK, we performed immunoprecipitation of the mutated receptor and investigated whether G-proteins were coassociated with the mutant.

The WT-CCKBR Stimulates Total Inositol Phosphate Production via Gα Proteins—To date no study has reported direct association of the WT-CCKBR with a G-protein. A previous study on COS-7 cells expressing the WT-CCKBR showed that CCK-stimulated PLC activation was pertussis toxin-insensitive suggesting that the WT-CCKBR is coupled to a G-protein of the G2 family (4). In order to verify the coupling of the WT-CCKBR to Gα proteins, COS-7 cells were cotransfected with pCDL-SRα-WT-CCKBR and increasing concentrations of pCIS-Gα and assayed for total IP production after stimulation with 10⁻⁶ M CCK. Coexpression of Gα with the WT-CCKBR increased IP production in a concentration-dependent manner that reached a maximum when cells were transfected with 2 μg of pCIS-Gα (Fig. 8). In similar conditions, coexpression of Gα with the N391A-CCKBR mutant did not induce any IP production indicating that the mutant does not stimulate IP production even when Gα subunits are overexpressed (not shown).

These results confirm that the WT-CCKBR couples to Gα proteins, and thus in the subsequent experiments, we looked for the presence of Gα proteins in the immunoprecipitates.

Association of the Inactive N391A-CCKBR Mutant with Gα Subunits in Response to CCK—Immunoprecipitation of the wild type and mutant receptors was performed using an antibody directed against the hemagglutinin epitope (HA) fused to the amino terminus of the WT-CCKBR and N391A-CCKBR mutant transiently expressed in COS-7 cells. We verified that epitope-tagged wild type (HA-WT-CCKBR) and mutant (HA-N391A-CCKBR) receptors expressed in COS-7 cells displayed similar CCK affinities, maximal binding capacities, and IP production compared with untagged wild type and mutated receptors (not shown). In addition, to confirm that the transfection and expression efficiencies of both tagged receptors were similar, we performed immunoblotting of COS cells lysates expressing HA-tagged wild type and mutated receptors with anti-HA antibodies. The CCKBR was expected to migrate at a mass of 68–97 kDa as determined previously with a photoactivatable CCK probe in COS cells expressing the CCKBR (30). As shown in Fig. 9, equal amounts of HA-WT-CCKBR and HA-N391A-CCKBR migrating at a mass of 85 kDa were revealed (lanes 1 and 2, respectively), and in non-transfected cells no corresponding band was detected (lane 3).

The Gα subunits associated with immunoprecipitated receptors were visualized by Western blotting with a well characterized, specific, and high affinity-purified polyclonal antibody raised against the carboxyl-terminal decapeptide of Gα11α subunits (31). We determined whether the Gα/G11α antibodies were able to detect endogenous Gα/G11α subunits by performing Western blot analysis on non-transfected COS-7 cells lysates. No endogenous Gα/G11α subunits were detected with the Gα/G11α antibodies (Fig. 10, lane 1). These results are consistent with a previous study reporting that COS-7 cells express low amounts of endogenous Gα/G11α subunits (32). In contrast, when COS-7 cells were transfected with 2 μg of pCIS-Gα, Gα subunits were detected by Gα/G11α antibodies migrating at a mass of 42 kDa (Fig. 10, lane 2). Thus, to permit visualization of receptor-Gα complexes, COS-7 cells were co-transfected with plasmids coding for Gα subunits and either HA-WT-CCKBR or HA-N391A-CCKBR mutant.

We first examined the association of HA-WT-CCKBR with Gα subunits. COS-7 cells coexpressing HA-WT-CCKBR and Gα subunits were stimulated with 10 nM CCK, a concentration that induces maximal inositol phosphate response (Fig. 1). The cells were then solubilized and subjected to immunoprecipitation with anti-HA antibodies. As shown in Fig. 11, upper panel, when cells coexpressing Gα subunits and HA-WT-CCKBR were treated with CCK, Gα subunits were coimmunoprecipitated with the HA-WT-CCKBR and identified after reaction with the anti-Gα/G11α antibodies as a band that migrated at 42 kDa. In contrast, in CCK-untreated cells, a low basal association between Gα subunits and HA-WT-CCKBR was observed. The effect of CCK on Gα/HA-WT-CCKBR complex formation was 2.8-fold ± 0.8 of the basal level (mean ± S.E. of five experiments). As control, non-transfected cell lysates were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-Gα/G11α antibodies. Under these conditions, no band corresponding to the Gα subunits was detected (Fig. 11, upper panel). These results revealed that the wild type receptor coupled specifically to Gα subunits when stimulated by CCK.
The mean mutant N391A receptors were incubated with 50 pm 125I-BH-CCK-9 in the presence of increasing concentrations of GTPγS. Data are expressed as percent of maximum specific bound 125I-BH-CCK-9 and represent the mean ± S.E. of three experiments performed in duplicate.

FIG. 6. GTPγS effect on 125I-BH-CCK-9 binding to the WT-CCKBR and N391A-CCKBR mutant transiently expressed in COS cells. Membranes from COS-7 cells expressing the wild type or mutant N391A receptors were incubated with 50 pm 125I-BH-CCK-9 in the presence of either 30 mM NaCl plus 10 μM AlCl3 (close bars) or 30 mM NaF plus 10 μM AlCl3 (open bars). Data are presented as percent of specific binding in medium containing 30 mM NaCl plus 10 μM AlCl3 (control) and represents the mean ± S.E. of three experiments performed in duplicate.

FIG. 7. 125I-BH-CCK-9 binding to COS cells transiently expressing the WT-CCKBR or N391A-CCKBR mutant. COS-7 cells transiently expressing the WT-CCKBR or N391A-CCKBR mutant were incubated with 50 pm 125I-BH-CCK-9 in the presence of either 30 mM NaCl plus 10 μM AlCl3 (close bars) or 30 mM NaF plus 10 μM AlCl3 (open bars). Data are presented as percent of specific binding in medium containing 30 mM NaCl plus 10 μM AlCl3 (control) and represents the mean ± S.E. of three experiments performed in duplicate.

FIG. 8. Effect of coexpression of Gα and WT-CCKBR in COS cells on CCK-stimulated total IP production. COS-7 cells were transfected with 6 μg of pcDNA3-SRα-WT-CCKBR and either increasing amounts of pcDNA3-Gα, or parental pcDNA3 vector and assayed for total IP production after stimulation by 10−8 M CCK-9 for 15 s. Results are expressed as percent of CCK-stimulated maximal IP production in cells cotransfected with pcDNA3-SRα-WT-CCKBR and parental pcDNA3 vector. The figure is the mean ± S.E. of four experiments performed in duplicate.

Fig. 9. Immunoblot analysis of HA-WT-CCKBR and HA-N391A-CCKBR mutant expressed in COS cells using anti-HA antibodies. Lysates (50 μg of proteins) of COS-7 cells transfected with 6 μg of pcDNA3-SRα-WT-CCKBR (lane 1), pcDNA3-SRα-N391A-CCKBR (lane 2), or from non-transfected COS-7 cells (lane 3) were immunoblotted with anti-HA antibodies. A representative autoradiogram from two independent experiments is shown.

FIG. 10. Immunoblot analysis of endogenous and transient expression of Gα subunits in COS cells using anti-Gα1α antibodies. Lysates from non-transfected COS-7 cells (lane 1) or from COS-7 cells transfected with 2 μg of pcDNA3-Gα plasmid (lane 2) were immunoblotted (ib) with anti-Gα1α antibodies.

FIG. 11. Association of the inactive N391A-CCKBR mutant with Gα subunits in response to CCK. COS-7 cells coexpressing Gα and either HA-WT-CCKBR (WT) or HA-N391A-CCKBR mutant (N391A) were stimulated (+) or not (−) with 10 nM CCK-9 for 15 s. Upper panel, cells lysates were immunoprecipitated (Ip) with anti-HA antibodies and immunoblotted (ib) with anti-Gα1α antibodies. Lower panel, after protein transfer, the top of the membrane was cut and immunoblotted with anti-HA antibodies to ensure equal amounts of immunoprecipitated wild type and mutated receptors. As control, non-transfected cells (NT) were immunoprecipitated with anti-HA antibodies and immunoblotted with anti-Gα1α antibodies (upper panel) or anti-HA antibodies (lower panel). Representative autoradiograms from five independent experiments are shown.

Next, we performed similar experiments with COS-7 cells coexpressing the HA-N391A-CCKBR mutant and Gα subunits. When cells were stimulated with 10 nM CCK and subjected to immunoprecipitation with anti-HA antibodies, Gα subunits coimmunoprecipitating with the HA-N391A-CCKBR mutant were revealed with anti-Gα1α antibodies (Fig. 11, upper panel). A low basal association between Gα subunits and HA-N391A-CCKBR was observed in CCK-untreated cells as with the wild type receptor. The effect of CCK on Gα1α-HA-N391A-CCKBR complex formation was 4.3-fold ± 1.1 of the basal level (mean ± S.E. of five experiments). These results suggest that the mutated receptor forms stable complexes with Gα subunits when stimulated by CCK. In parallel, to verify the amounts of immunoprecipitated wild type and mutated receptors, the top of the membrane was cut after protein transfer and separately immunoblotted with anti-HA antibodies. As shown in Fig. 11, lower panel, equal amounts of epitope-tagged wild type and mutated receptors migrating at a mass of 85 kDa were immunoprecipitated in all assays except in non-transfected cells where no corresponding band was detected.

Together these data suggest an essential and specific role of Asn-391 in the activation process of Gα proteins since the N391A-CCKBR mutant retains the capacity to interact with Gα1α proteins in response to CCK.

DISCUSSION

According to the importance of the Asn/Asp residue of the highly conserved (N/D)PX2–3Y motif in the function of several
G-protein-coupled receptors (7, 9–13), we have investigated whether a similar role could be attributed to the Asn-391 of the NPXY motif of the CCKBR. Mutation of this residue to Ala had no effect on agonists binding since the N391A-CCKBR mutant conserved a high affinity for CCK-9, CCK-4, and gastrin 17-I indicating that Asn-391 is not involved in agonist-binding sites. Moreover, this mutation produced a gain of affinity of 3- and 10-fold for the selective CCKBR antagonists, L365-260 and PD135-158. Because Asn-391 is located deep inside the transmembrane domain seven, it is likely that this residue is not directly involved in antagonist-binding sites. However, despite the high affinity binding displayed by CCK and selective agonists and antagonists for the mutant, this mutation completely abolished phosphoinositide hydrolysis and MAPK activation. This was not due to a defect of expression of the mutant at the cell surface since similar expression levels were measured for the mutant and wild type receptors.

We have shown in this study using the PKC-specific inhibitor bisindolylmaleimide GF109203X that 65% of CCK-induced p42 and p44 MAPK activation is PKC-dependent. Thus it appears that the remaining MAPK activation is independent of PKC and, accordingly, of PLC. These results are consistent with previous studies performed in other cell lines showing that both PKC-dependent and -independent pathways are involved in MAPK activation mediated by the CCKBR (5, 6). The fact that the mutation of Asn-391 inhibits at least two different signaling cascades without affecting the agonist-binding site suggests that this residue has a critical role in the CCKBR activation processes. These results are in agreement with the absence of effects of GTPγS and AIFF on CCK binding to the N391A-CCKBR mutant, whereas similar treatment reduced CCK binding to the WT-CCKBR. The reduction in CCK binding observed for the WT-CCKBR in the presence of GTPγS (or AlFF) is attributable to the dissociation of the receptor from GDPγS (or GDP-AlF) and βγ subunits and thus reflects the presence of G-proteins and their persistent activation. Absence of effects of GTPγS and AlF on CCK binding to the mutant receptor, in addition to its inactivation, could reflect an absence of G-protein association. Alternatively, the mutant could be associated to G-proteins, but the processes of guanine nucleotide exchanges and/or α and βγ subunits dissociation from the mutant receptor could be defective. Such a mechanism is supported by a previous study on the rhodopsin receptor showing that the mutation of two segments in the second and third intracellular loops produced mutants that normally bound Gt in response to light but failed to release the bound Gt in the presence of guanosine triphosphate (33). Moreover, these mutants were shown to bind the Gt αβγ-GDP form of Gt that was defective in catalyzing GDP release (34). In order to determine whether the N391A-CCKBR mutant retains the capacity to interact with G-proteins, we performed immunoprecipitation of the mutant receptor and investigated whether G-proteins were coassembled with the mutant by Western blotting. To date, such approach has not been used to evaluate if inactive mutants were coupled to G-proteins in other G-protein-coupled receptors. We looked for the presence of Gq proteins in the immunoprecipitates since we showed an increase in CCK-stimulated inositol phosphates response by coexpressing the WT-CCKBR with increasing amounts of Gqα subunits. Coupling of the WT-CCKBR to Gq proteins was further demonstrated by coimmunoprecipitating the WT-CCKBR with Gqα when cells were stimulated by CCK. In similar conditions, we found significantly more Gqα subunits associated with the mutated receptor than with the wild type CCKBR (4.3-fold ± 1.1 versus 2.8-fold ± 0.8 of the basal level), indicating that the mutated receptor forms stable complexes with Gqα subunits. These results suggest that the high affinity agonist state displayed by the mutated receptor is due to G-proteins association. In addition, the stability of the mutated receptor-Gq complexes supports that the inactivity of the mutant likely reflects a defect in the dissociation of α and βγ subunits and would be consistent with the absence of effect of GTPγS on CCK binding. However, the present study does not allow one to conclude about the state of the Gq proteins associated with the mutated receptor that could contained GDP or GTP bound to the α subunit or no nucleotides (empty state). Nevertheless, the fact that this mutation allows stable receptor-G-protein complexes to form but prevents Gq activation argues in favor of a specific role of Asn-391 in Gq protein activation. In agreement with the important role of Asn-391 in receptor activation, a computational modeling study has reported that the (N/D)P motif introduced a local flexibility in the seventh transmembrane domain of G-protein-coupled receptors that may play a role in receptor activation by functioning as a sensitive conformational switch (15). Consistent with this study, the activation of rhodopsin receptor has been correlated in a recent study with conformational changes at the cytoplasmic end of the seventh transmembrane helix by using an antibody directed against a part of the highly conserved NPXY motif. This antibody recognized specifically light-induced exposure of the epitope, whereas no binding was detected in the dark. The accessibility of the epitope to the antibody was correlated with the formation of the metarhodopsin II photointermediate, an active state that binds Gq and catalyzes guanine nucleotides exchanges. Based on these studies, it can be hypothesized that Asn-391 participates in CCKBR conformational changes that lead to Gq activation; however, a direct role of Asn-391 in Gq activation cannot be excluded.

In several G-protein-coupled receptors, this conserved Asn (Asp for the gonadotropin-releasing hormone receptor) was reported to interact with another highly conserved Asp (Asn for the gonadotropin-releasing hormone receptor) located in transmembrane domain 2 by performing reciprocal exchange of these two residues to restore activity of the mutants (11, 12, 35, 36). This was not the case in the CCKBR, since mutation of Asn-391 to Asp neither affected CCK binding nor PLC activation, whereas mutation of Asp-100 to Asn decreased by 50% the production of IP, leaving open the possibility of other interactions (4).

In this study, a PKC-independent pathway was found to couple the CCKBR to MAPK activation. However, the molecular entities involved in such a coupling are not well known. Occupancy of the CCKBR expressed in Rat-1 cells by CCK has been shown to elicit MAPK activation through a pathway that was independent of pertussis toxin-sensitive G-proteins and PKC but was dependent on p73Raf-1 (6). A βγ dimer of a pertussis toxin-insensitive G-protein acting on a Ras-dependent/ PKC-independent pathway was described to be involved in MAPK activation mediated by the muscarinic-1 receptor (29, 37). An implication of such βγ dimer in addition to α subunit would be consistent with the complete loss of PKC-dependent and -independent MAPK activation displayed by the N391A-CCKBR mutant.

In conclusion, in the present study we show that the N391A-CCKBR mutant, which lacks the ability to activate PLC and MAPK but binds agonists with high affinity, is able to form stable complexes with Gq in response to CCK, suggesting an essential and specific role of Asn-391 in the activation process of Gq proteins. More generally, these results show that the formation of high affinity agonist-receptor-Gq complexes is not sufficient to activate Gq and thus indicate that specific determinants of the receptor are involved in Gq activation, whereas...
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others are involved in G<sub>q</sub> binding. Since this Asn residue is highly conserved in G-protein-coupled receptors, it would be of interest to determine if a similar role could be extended to others receptors of this family.

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