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ACCELERATED COMMUNICATION

Mutation of Asp^{100} in the Second Transmembrane Domain of the Cholecystokinin B Receptor Increases Antagonist Binding and Reduces Signal Transduction

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SUMMARY

We examined the functional significance of two residues present in the second (Asp^{100}) and seventh (Asn^{391}) transmembrane domains of the rat cholecystokinin$_B$ (CCK$_B$) receptor that are highly conserved among the members of the G protein-coupled receptor family. Substitution of Asn for Asp^{100} by using site-directed mutagenesis did not change the affinity and selectivity for agonists but slightly increased the affinity of three CCK$_B$-selective antagonists of different chemical structures. Cells expressing the mutant receptor exhibited a 50% reduction in CCK$_8$-induced phosphoinositide turnover compared with cells expressing the wild-type receptor, suggesting a critical role for this residue in the coupling of the CCK$_B$ receptor to G protein. This latter was shown to be insensitive to pertussis toxin treatment and could therefore belong to the G$_q$ family. Replacement of Asn^{391} by Asp located in the seventh transmembrane domain did not change agonist binding or phosphoinositide turnover. This suggests that in contrast to the gonadotropin-releasing hormone receptor, there is no direct interaction in the CCK$_B$ receptor between Asp^{100} and Asn^{391}. However, a rhodopsin-based molecular modeling of the CCK$_B$ receptor showed a spatial proximity between Asp^{100} and the carboxyl terminal part of the third intracellular loop, known to interact with G protein. This could explain the reduction in phosphoinositide turnover observed with the Asn^{100} mutant.

The complementary DNAs encoding the two CCK receptors (CCK$_A$-R and CCK$_B$-R) have been cloned from various species (reviewed in Ref. 1). Their sequence analysis has shown that they belong to the GPCR superfamily. There is considerable interest in the development of CCK$_B$-R agonists, which could improve vigilance and memory processes (2), and of CCK$_B$-R antagonists, which have been reported to inhibit panic attacks triggered by administration of CCK$_4$ in humans (3), to potentiate opioid analgesia (4, 5), and to produce antidepressant-like effects in rodents (6). Several potent and specific CCK$_B$-R antagonists have been synthesized (reviewed in Ref. 7). However, most of these compounds have from poor water solubility and bioavailability (8). In contrast, no nonpeptide agonists have been reported as the only systemically active CCK$_B$-R agonist, BC 264, corresponds to a modified CCK$_8$ analogue (9). It would therefore be interesting to elucidate the specific molecular interactions involved in CCK$_B$-R agonist and antagonist binding with an aim of developing improved selective ligands.

The molecular processes by which agonists and antagonists bind to CCK-R are unknown. Nevertheless, the binding domain of CCK$_8$ and related small ligands is believed to be localized in the TM region of the receptor. Thus, eight residues located in the TM helices of the human CCK$_B$-R (10), as

ABBREVIATIONS: CCK, cholecystokinin; CCK-R, cholecystokinin receptor; CCK$_8$, cholecystokinin octapeptide; GPCR, G protein-coupled receptor; PI, phosphoinositide; PTX, pertussis toxin; TM, transmembrane domain; WT, wild-type; A-71623, Boc-Trp-Lys(Tac)-Asp-MePhe-NH$_2$; L-364,718, 3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3yl)-1*H*-indole-2-carboxamide; L-365,260, 3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3yl)-*N'*-(3-methylphenyl)urea; pBC 264, propionyl-Boc-Tyr(SO$_3$H)-gNle-mGly-Trp-(NMe)-Nle-Asp-PheNH$_2$; PD-134,308, 4-{[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.1^{3,7}]dec-2yloxy)carbonyl]aminopro-pyl]amino]-1-phenylethyl]amino}-4-oxo-[*R*-(*R**,*R**)]-butanoate-*N*-methyl-D-glucamine; SR-27,897B, 1-{[2-(4-(2-chlorophenyl)thiazol-2-yl)aminocarbonyl]indolyl}acetic acid; WP-69, Boc-Trp-Phg-Asp-Nal-*N*(Me)$_2$; PLC, phophoslipase C.

well as a His residue located in TM-VII of the rat CCK_B-R (11), have been suggested to be involved in selectivity of CCK_B versus CCK_A antagonists. Residues highly conserved among all GPCRs are likely to be essential structural determinants of receptor function. One such residue is an Asp located in TM-II, which is 98% conserved (12). Replacement of this residue in different receptors has been found to produce a variety of functional effects, such as reduction in agonist affinity (13–17); loss of binding modulation by pH (17), sodium (17, 18), or GTP analogues (16); and alteration or complete elimination of transduction processes involving G protein coupling (16, 20). Interestingly, in the gonadotropin-releasing hormone receptor, an Asn residue (Asn^{87}) is located in the TM-II at the position of this highly conserved Asp residue, whereas an Asp residue (Asp^{318}) is present in the TM-VII at the position where nearly all other GPCRs have an Asn. These two residues were supposed to be adjacent in space because replacement of Asn^{87} by Asp eliminated agonist binding, whereas the reciprocal double mutation restored binding (21). In the rat CCK_B-R, the Asn residue located in the TM-VII (Asn^{391}) could also be involved in a direct interaction with Asp^{100} of the TM-II. In the present study, with the use of site-directed mutagenesis, Asp^{100} of the rat CCK_B-R was replaced by an Asn residue (D100N), and Asn^{391} was changed for an Asp residue (N391D). The WT and mutated CCK_B-Rs were transiently expressed in Cos-7 cells, and radioligand binding and inositol phosphates assays were performed. The mutation of Asp^{100} to Asn had no effect on agonist binding but slightly improved the affinities of CCK_B antagonists. We also found a 50% reduction in PI hydrolysis with this mutant, suggesting that Asp^{100} plays a critical role in the coupling of CCK_B-R with the G protein. Surprisingly, the mutation of Asn^{391} had no effects on either CCK_8 binding or second-messenger production. A model of the CCK_B-R based on rhodopsin structure appears to indicate a spatial proximity between Asp^{100} and the carboxyl terminal region of the third intracellular loop, which could account for the loss of G protein coupling in the D100N mutant receptor. Finally, our results clearly demonstrated that the rat CCK_B-R is coupled to a PTX-insensitive G protein.

Materials and Methods

Reagents. CCK_4 was purchased from Bachem (Buhendorf, Switzerland). CCK_8, pBC 264, A-71,623, L-365,260, L-364,718, PD-134,308, and WP-69 were synthesized in the laboratory according to previously reported procedures (9, 22–26). SR-27,897B was generously provided by Sanofi (Toulouse, France). Radiolabeled compounds, such as [α-^{33}P]dATP (specific activity, 1000–3000 Ci/mmol), [^{3}H]pCCK_8 (specific activity, 60–90 Ci/mmol), and myo-[2-^{3}H]inositol (specific activity, 60–90 Ci/mmol) were purchased from Amersham (Les Ulis, France). Cell culture reagents were obtained from GIBCO-BRL (Cergy, France). PTX was purchased from Sigma Chemical Co. (Paris, France)

Site-directed mutagenesis. The cDNA of the rat CCK_B-R was obtained as previously described (27) and inserted into the expression vector pcDNA3 (11). Two oligonucleotides of 21 bp were designed to replace the codon for Asp (GAC) located at amino acid position 100 with a codon for Asp (5′-GGCAGTCAGCAACCTCCTGCT-3′) and to replace the codon for Asp (AAC) located at amino acid position 391 with a codon for Asp (5′-TGCTTGTGTCGACCCCCTGGT-3′). Double-strand mutagenesis was carried out as previously described (11). Authenticity of the mutations was confirmed by sequencing over the entire protein-coding region with the Sequenase version 2.0 DNA sequencing kit (US Biochemical) and [α-^{33}P]dATP.

Cell culture and radioligand binding assays. Cos-7 cells, which do not express CCK_B-Rs, were grown and transfected as previously described (11). At 48 hr after the transfections, the binding assays were performed directly on cells in Dulbecco's modified Eagle medium containing 5 mM $MgCl_2$ and 0.2 mg/ml bacitracin. Each assay was performed in a final volume of 0.5 ml. Incubations were carried out for 90 min at 25°. For saturation binding experiments, the concentration of [^{3}H]pCCK_8 varied from 50 to 6000 pM. For competition experiments, a fixed concentration of 500 pM [^{3}H]pCCK_8 was used in the presence of various concentrations of the competitor. Nonspecific binding was determined with 1 μM CCK_8 and was found to be <20% of maximum binding at saturation in all experiments. Incubations (90 min at 25°) were stopped by the removal of the media, followed by two washes with 1 ml of phosphate-buffered saline. To harvest the cells, 200 μl of trypsin was added to each well. After incubation at 37°, the cells were collected, and radioactivity was determined. Saturation binding parameters for [^{3}H]pCCK_8 (i.e., K_d, B_{max}) were determined with the computer program EBDA. K_i values were determined with the Cheng-Prussof equation: $K_i = IC_{50}/[1 + (\text{radioligand concentration}/K_d \text{ of the radioligand})]$.

Inositol phosphate assays. Cos-7 cells transiently expressing WT and mutated CCK_B-Rs were assayed for CCK_8-stimulated PI hydrolysis, essentially as previously described (28). Before assays, transfected cells were grown in the presence of 1 μCi/ml myo-[2-^{3}H]inositol for 16 hr at 37°. Cells were treated with 10 mM LiCl for 30 min at 37°, and various concentrations of CCK_8 were added to the cells. After 45 min at 37°, the incubation medium was removed, and the cells were washed twice with 1 ml of phosphate-buffered saline. The reaction was stopped by the addition of 400 μl of ice-cold 75% methanol and 300 μl of 0.12% Triton. The cells were scraped, and the suspension was subjected to chloroform extraction. Then, 0.5 ml of the aqueous phase was added to 4.5 ml H_2O. The solution was loaded onto a 0.5-ml column containing AG1-X8 Dowex anion exchange resin (Bio-Rad, Ivry-sur-Seine, France). The column was washed with 1 ml of distilled water followed by 5 ml of 5 mM sodium borate/60 mM sodium formate. Total [^{3}H] inositol phosphates were then eluted into scintillation vials with 5 ml of 1 M ammonium formate/0.1 M formic acid. After the addition of scintillation mixture, radioactivity was counted. The mean maximum inositol phosphate level obtained from dose-response curves for mutant receptors was expressed as a percentage of the inositol phosphate production by the WT receptor in the same experiment.

PTX inhibition assays. To characterize the effect of PTX on agonist-stimulated PI hydrolysis, transfected cells labeled with myo-[2-^{3}H]inositol were treated with concentrations of PTX ranging from 0 to 100 ng/ml for 16 hr at 37° before treatment with an agonist. Inositol phosphate assays were then performed as described.

Computer modeling. The molecular model of the WT CCK_B-R was constructed with the TM helical positions found in the bacteriorhodopsin crystal structure as a starting point of the optimization procedures and used successively the human rhodopsin and the β_2-adrenergic receptor for sequence alignment procedures (30). Next, all extracellular and intracellular loops connecting the transmembrane helices were added to the preliminary 7-helix bundle and then modeled with the use of a simulated annealing procedure. The entire system was relaxed, with possible translation and rotation movements of individual TM helices taken into account. Details of the building of these models will be presented elsewhere.[1] The homology, energy minimization, and dynamics steps were produced with the use of the Biosym molecular modeling software. Molecular modeling was performed independently from the experimental work to avoid any bias in subsequent analysis of the results.

[1] N. Goodreau, A. Jagerschmidt, B. P. Roques and B. Maigret, unpublished observations.

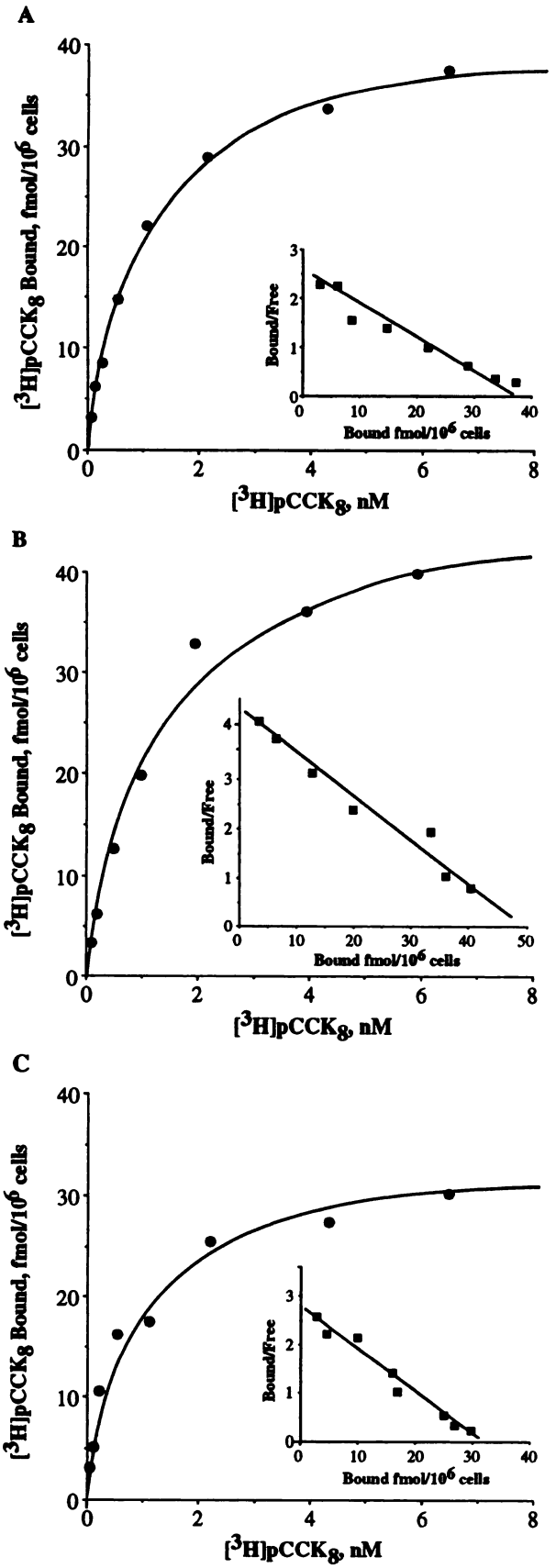

Fig. 1. Saturation analysis of [^{3}H]pCCK$_8$ binding to WT and mutant rat CCK$_B$-Rs expressed in Cos-7 cells. A, Specific [^{3}H]pCCK$_8$ binding to WT receptors. B, Specific [^{3}H]pCCK$_8$ binding to D100N receptors. C, Specific [^{3}H]pCCK$_8$ binding to N391D receptors. *Insets*, Scatchard transformation of the data. Assays are described in Materials and Methods. The experimental curves are representative of three assays performed in triplicate. In these particular experiments, the binding parameters for N391D were K_d = 1.2 nM and B_{max} = 39 fmol/10^6 cells; K_d = 1.2 nM and B_{max} = 44 fmol/10^6 cells for N391D; and K_d = 0.5 nM and B_{max} = 30 fmol/10^6 cells.

Results

To examine the function of both Asp and Asn residues located in TM-II (Asp^{100}) and TM-VII (Asn^{391}) of the rat CCK$_B$-R, site-directed mutagenesis experiments were performed. WT and mutated receptors were transiently expressed in Cos-7 cells, and ligand binding parameters were determined. As a measure of receptor activation, inositol phosphate assays were also performed.

Ligand binding. As shown in Fig. 1, the binding of [^{3}H]pCCK$_8$ to transfected cells was specific and saturable. The B_{max} values for the WT (37 ± 10 fmol/10^6 cells) and D100N (38 ± 20 fmol/10^6 cells) receptors were almost identical, whereas that of the N391D mutant receptor was slightly decreased (20 ± 9 fmol/10^6 cells). These results indicated that the expression and membrane localization of the receptors were not altered by these mutations. No binding activity was detected in untransfected cells (not shown). Transient expression of the receptor in Cos-7 cells, which involved the transfection of a new batch of cells for each separate experiment, could explain the relatively high standard error for B_{max} values. Scatchard analysis of several binding isotherms showed the occurrence of a single class of binding sites, with similar affinity values for WT (K_d = 1.03 ± 0.30 nM) and D100N mutant (K_d = 1.23 ± 0.20 nM) receptors, whereas a 2-fold higher affinity was noted for N391D mutant receptor (K_d = 0.54 ± 0.07 nM). These results indicated that Asp^{100} and Asn^{391} were not involved in the binding of the natural peptide ligand CCK$_8$. Agonist binding to WT and D100N mutant receptors was studied by competition experiments using [^{3}H]pCCK$_8$ as radioligand. Four compounds were studied: CCK$_8$; pBC264, a highly potent and selective CCK$_B$-R ligand derived from CCK$_8$ (9); CCK$_4$; and A-71,623, a CCK$_A$-R-selective agonist derived from CCK$_4$ (22). The K_i values of these compounds were found to be similar for both WT and D100N mutant receptors (Table 1), with a rank order of potency as follows: pBC264 > CCK$_8$ > CCK$_4$ > A-71,623, which is in agreement with values reported in the literature for rat brain membranes CCK$_B$ receptor (22, 31). These results demonstrated that the Asp^{100} residue is not involved in the agonist binding domain of the CCK$_B$-R. The affinities of three CCK$_B$-R-selective antagonists (L-365,260, PD-134,308, and WP-69) and two CCK$_A$-R-selective antagonists (L-364,718 and SR-27,897B) were also determined. In contrast to agonist binding, which was unaffected by the mutation, the K_i values of all three CCK$_B$-R-selective antagonists were reduced. The affinities of the benzodiazepine-based antagonist L-365,260 (K_i = 1.09 nM) and the peptoid antagonist PD-134,308 (K_i = 0.78 nM) for the D100N mutant receptor were 6-fold higher than those for the WT receptor (L-365,260, K_i = 5.96 nM; PD-134,308, K_i = 4.39 nM). A 4-fold increase in the modified peptide WP-69 affinity was also observed (Table 1). Interestingly, replacement of Asp^{100} with Asn had different effects on the CCK$_A$-R-selective antagonists, depending on the compound tested. This substitution led, as for CCK$_B$-R antagonists, to a 5-fold in-

TABLE 1

Comparison of pharmacological properties of WT and D100N mutant CCK_B-R

K_i values were calculated from competition binding experiments, using [^{3}H]pCCK_8 (0.5 nM) as radioligand, as described in Materials and Methods.

Ligand	K_i	
	WT	D100N
	nM	
Agonists		
CCK8	1.10 ± 0.33	0.80 ± 0.20
pBC 264	0.58 ± 0.11	0.63 ± 0.10
CCK_4	17.71 ± 1.35	19.90 ± 3.72
A-71,623	725 ± 77	1111 ± 208
Antagonists		
L-365,260	5.96 ± 0.14	1.09 ± 0.37[b]
PD-134,308	4.39 ± 2.03	0.78 ± 0.01[a]
WP-69	26.42 ± 8.06	6.20 ± 2.30[a]
L-364,718	68 ± 15	82 ± 6
SR-27,897B	237 ± 57	44 ± 11[b]

Data are expressed as the mean ± standard error of three or four experiments, each performed in triplicate.
[a] $p < 0.05$
[b] $p < 0.01$ (Student's t test).

crease in SR-27,897B affinity (K_i = 237 and 44 nM for WT and D100N receptors, respectively), whereas it had little or no effect on the affinity of L364,718 (K_i = 68 and 82 nM for WT and D100N receptors, respectively).

PI turnover. Rat CCK_B-Rs expressed in Cos-7 cells display agonist-mediated dose-dependent increases in PI hydrolysis (32). The addition of CCK_8 to Cos-7 cells expressing the WT CCK_B-R produced a linear increase in the formation of radiolabeled inositol phosphates between 0 and 60 min (not shown). As previously described for the α-adrenergic receptor subtypes (33), the CCK_B-R number expressed by the cells was shown to increase with the amount of DNA used during the transfections. In our experiments, the maximum level of receptor number was reached for plasmid DNA concentrations ranging from 6 to 10 $\mu g/10^5$ cells (Fig. 2). In this concentration range, there were similar levels of expression for WT and D100N receptors. Thus, the results obtained concerning PI turnover cannot be attributed to a decrease in expression of the mutated receptor. Furthermore, the maximum level of PI hydrolysis was also found to be highly dependent on receptor density (not shown). Therefore, to compare the inositol phosphate levels produced by both WT and mutated receptors, we used 8 μg DNA/10^5 cells for transfection, which led in both cases to a final receptor expression level of 800 fmol/10^6 cells. Moreover, saturation experiments have shown that regardless of the amount of DNA used to transfect the cells, the binding parameters of [^{3}H]pCCK_8 other than B_{max} were similar (not shown). In Cos-7 cells expressing the WT CCK_B-R, 1 μM CCK_8 generated an 8-fold increase in inositol phosphate level compared with the level in cells not exposed to CCK_8. No change in PI hydrolysis was observed in control cells transfected with pcDNA3 alone. In contrast, PI turnover induced by CCK_8 was markedly attenuated with the D100N mutant receptor (Fig. 3) as the mutation resulted in a 50% impairment in the ability of CCK_8 to activate PI metabolism. This decrease was accompanied by a significant 2-fold reduction in CCK_8 efficacy (EC_{50} = 4.53 ± 2.81 nM and 11.91 ± 0.97 nM for WT and D100N, respectively), whereas the binding affinity of CCK_8 remained unmodified (as discussed). In contrast, in cells expressing the N391D mutant receptor, CCK_8 activated PI hydrolysis with the same potency (Fig. 3) and efficacy (EC_{50} = 5.65 ± 0.65 nM) as the WT receptor. Based on results obtained with both D100N and N391D mutant receptors, it appears that only the Asp^{100} residue has an important role in the interaction of the CCK_B-R with its G protein-effector associated system.

PTX inhibition assays. PTX was used to characterize the G protein(s) involved in the coupling of the rat CCK_B-R to a phospholipase generating PI hydrolysis. The effect of PTX on PI metabolism in Cos-7 cells was studied for both WT and D100N mutated receptors. As shown in Fig. 4, PTX (at concentrations up to 100 ng/ml) was unable to block CCK_8-mediated PI hydrolysis in both cases. On the basis of this

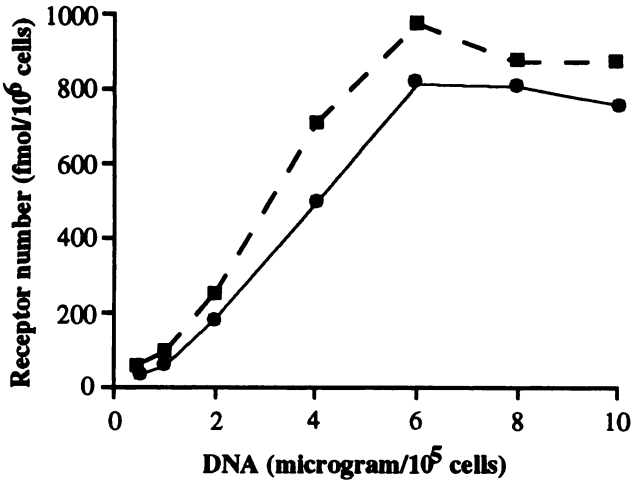

Fig. 2. Expression of CCK_B receptors in Cos-7 cells. Cos-7 cells were transfected with increasing amounts of recombinant plasmid DNA encoding either the WT (●) or D100N (■) mutant receptors. Receptor concentrations were determined by ligand binding using 5 nM [^{3}H]pCCK_8. The results represent measurements obtained from two experiments performed in triplicate.

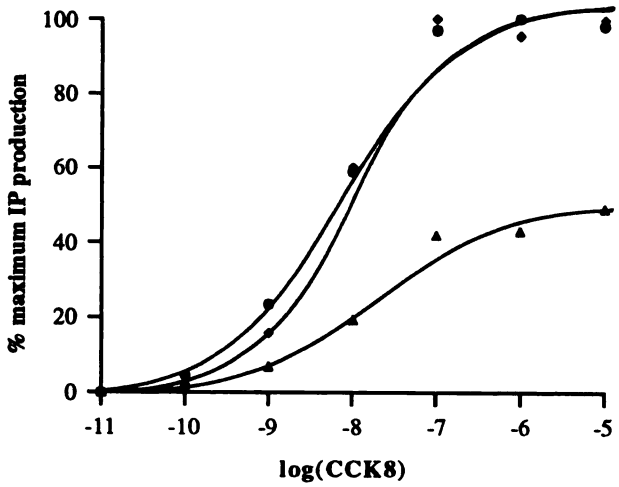

Fig. 3. CCK_8 stimulation of PI hydrolysis. Cos-7 cells expressing WT (●), D100N (▲), or N391D (♦) mutant rat CCK_B-R were treated as described in Materials and Methods. Data points represented are mean values of triplicate determinations in three different experiments. There was no CCK_8-stimulated PI hydrolysis in untransfected Cos-7 cells. For comparison, the maximum amount of [^{3}H]inositol phosphate accumulation induced by WT CCK_B-R, in a typical experiment, was 1154 cpm, with a basal accumulation of 147 cpm.

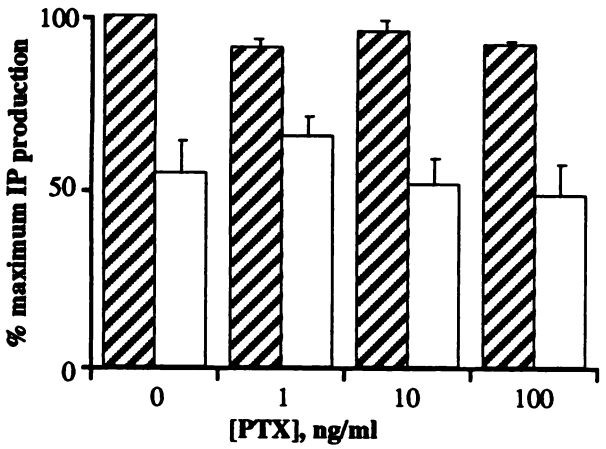

Fig. 4. PTX sensitivity of CCK_B-induced PI turnover. Cos-7 cells expressing either WT (*striped bars*) or D100N (*open bars*) CCK_B-R were treated with increasing PTX concentrations as described in Materials and Methods. Histograms represent the mean of two independent experiments.

result, it appears that the rat CCK_B-R, when expressed in Cos-7 cells, is coupled to a PTX-insensitive G protein.

Discussion

An Asp residue located in the TM-II is conserved among most of the GPCRs (12). The exceptionally high conservation of this negatively charged amino acid suggests that it could have a critical role in the structural organization and in the functioning of GPCR.

In the present study, we showed that the mutation of Asp^{100} for Asn in the rat CCK_B-R does not alter the binding of CCK_B or CCK_A agonists. This indicates that in the rat CCK_B-R, the Asp residue located in the TM-II is not critically involved in the interaction of agonists and that the mutation probably does not modify the spatial arrangement of the agonist binding site (Table 1). Similar results were also observed after mutation of the corresponding Asp residue in the α_2-adrenergic (15), m1 muscarinic acetylcholine (14), and angiotensin II (20) receptors. However, the corresponding mutation in the opioid δ receptor reduced the affinity of agonists without changing the transduction processes (13), whereas for the $serotonin_2$ receptor, both parameters were altered (16).

In contrast to our finding with agonists, abolition of the negative charge in the TM-II of the rat CCK_B-R slightly improved the affinity of the various CCK_B antagonists used (Table 1). Interestingly, the three tested CCK_B-R antagonists corresponded to molecules belonging to different chemical classes (L-365,260 is a benzodiazepine-derived compound, PD-134,308 is a CCK_4-based peptoid, and WP-69 is a modified peptide ligand). Furthermore, at pH 7, PD-134,308 and WP-69 should be negatively charged, whereas L-365,260 probably is positively charged due to protonation of the benzodiazepine moiety. Consequently, the improved affinity of these molecules for the D100N receptor cannot be related to a reduction in charge repulsion. This suggests that there probably are no direct interactions between the CCK_B-R antagonists and the Asp^{100} residue of the receptor. Accordingly, data obtained from a computer-generated model of the CCK_B-R indicate that the Asp^{100} residue probably is located at the bottom of the TM domain of the receptor (Fig. 5) and thus is inaccessible to agonists and antagonists whose binding sites appear to be located in the top half of the TM domain (10, 11). Therefore, the observed improved affinity of antagonists for the mutant receptor could very likely be related to a discrete and thermodynamically favorable conformational change of the CCK_B-R antagonist binding site in the D100N receptor. This supports the hypothesized existence in all GPCRs of structurally different interconverting states for agonists and antagonists (34). Moreover, the results indicate that all of the studied CCK_B-R antagonists might fit a common binding area within the CCK_B-R. Accordingly, a comparative conformational analysis of two of these antagonists (L-365, 260 and WP-69) has shown that although the origins and development of these compounds were completely independent, they have remarkable spatial overlapping (32). With respect to the CCK_A-R-selective antagonists SR-27,897B and L-364,718, it appears that different residues of the CCK_B-R might be involved in the binding of these two ligands because the mutation led to opposite results: improved affinity for SR-27,897B and no significant change for L-364,718. Further conformational analysis and receptor docking experiments will be necessary to explain these findings.

In addition, results of the present study confirm that the CCK_B-R antagonist L-365,260 and the CCK_A-R antagonist L-364,718, both benzodiazepin-derived ligands, interact specifically with different residues within the CCK_A and CCK_B receptors. The D100N mutation improved the affinity of L-365,260 for the rat CCK_B-R without changing that of

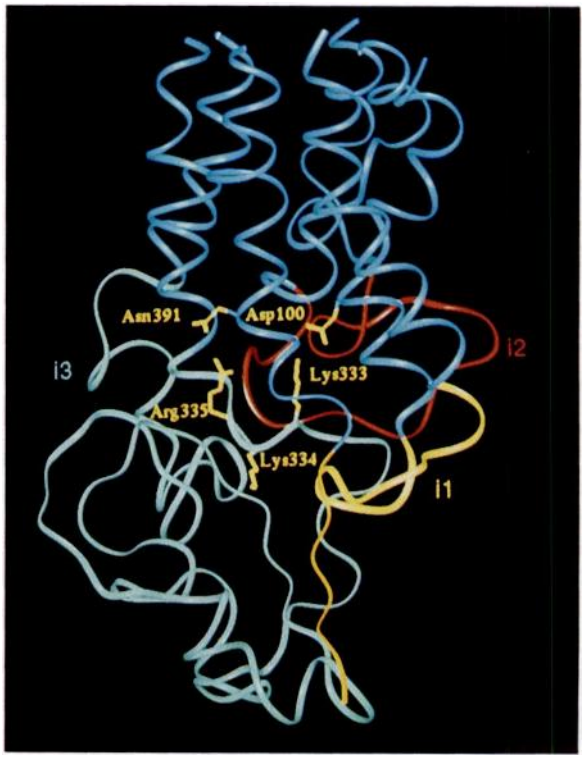

Fig. 5. Stereoview of the computer-generated model of the CCK_B-R showing longitudinal views of the TM helices (*blue*); the intracellular domains i1 (*yellow*), i2 (*red*), and i3 (*green*); and the carboxyl terminal part (*orange*). Asp^{100}, Asn^{391}, and the basic residues of the carboxyl terminal part of the third intracellular loop, Lys^{333}, Lys^{334}, and Arg^{335}, are represented in *yellow*.

L-364,718. This result is reminiscent of recent demonstrations by site-directed mutagenesis that showed that the replacement of His^{381} by a Leu residue in the CCK_B-R led to an improvement in the affinity of L-364,718 without changing the affinity of L-365,260 (10, 11). Agonist stimulation of the CCK_B-R led to inositol phosphate production as previously demonstrated either in cells expressing endogenous CCK_B-R, such as rabbit gastric parietal cells (35) or GH3 cells (37), or in transfected Cos-7 cells (32). The present study shows for the first time that in the CCK_B-R, Asp^{100} is involved in CCK_8-stimulated second messenger production. The maximum increase in inositol phosphate production was reduced by 50% with the D100N mutant receptor compared with the WT receptor (Fig. 4). The decrease in efficiency of second messenger formation cannot be attributed to either a loss of agonist affinity because CCK_8 binds to WT and D100N receptors with similar K_d values (Fig. 1) or to differences in receptor expression because similar amounts of binding sites were found in cells expressing WT or D100N receptors (Fig. 2). Thus, the decrease in PI hydrolysis observed with the mutant indicates that although not directly involved in the agonist binding, Asp^{100} could be one of the residues implicated in transduction processes. It is interesting to note that a similar conclusion was recently reported for the angiotensin II receptor (20). The replacement of Asp^{100} by Asn reduces the ability of the receptor to generate the second messenger, probably because the receptor becomes less sensitive to agonist-induced activation. PLC-β and PLC-ϵ, which are responsible for agonist-stimulated PI turnover, have been shown to be G protein regulated (38). The GPCRs that have been identified show differential PTX sensitivity. To determine whether the G protein associated with the CCK_B-R was sensitive to this toxin, inositol phosphate assays were performed after PTX treatment (Fig. 4). Our results clearly show that the rat CCK_B-R, when expressed in Cos-7 cells, is coupled to a PTX-insensitive G protein. Moreover, because it is well known that a large proportion of the PTX-insensitive G proteins that regulate PLC activity belong to the G_q family (for review, see Ref. 38), it is tempting to assume that the CCK_B-R is coupled to one of these G_q proteins. In most of the three-dimensional models that have been proposed for GPCRs, TM-II and TM-VII are found adjacent in space (for review, see Ref. 37). It was recently suggested, in the case of the gonadotropin-releasing hormone receptor, that this interhelical proximity resulted in an interaction, probably through hydrogen bonding, between the side chains of the Asn^{87} residue in TM-II and the Asp^{318} residue in TM-VII (21). It was hypothesized that this kind of interhelical interaction might be extended to the other GPCRs. Therefore, in an attempt to describe CCK_B-R functioning at the molecular level, we generated a three-dimensional model of this receptor. In this model, the side chains of the Asp^{100} and Asn^{391} are not oriented in the same direction as Asn^{87} and Asp^{318} in the gonadotropin-releasing hormone receptor (21). This finding could explain our experimental results because no change in either CCK_8 affinity or PI turnover was observed in the N391D mutant receptor when compared with the WT receptor. If Asp^{100} and Asn^{391} are involved in electrostatic or hydrogen bonding interactions, the replacement of Asn^{391} by Asp would result in an electrostatic repulsion that would, in turn, impair ligand binding and/or second messenger activation through modification of the receptor structure. Nevertheless, as mentioned, replacement of Asp^{100} by Asn resulted in a 50% reduction in PI turnover. Due to the absence of change in CCK_8 affinity and binding site levels in the D100N mutant, the 50% reduction in IP formation could be due to a lower intrinsic activity of the receptor, a pharmacological concept generally used to characterize agonist potency. In our CCK_B-R model, the side chain of Asp^{100} was found to point in the direction of a cluster of basic residues located in the carboxyl terminal portion of the third intracellular loop (Fig. 5). Interestingly, in most of the GPCRs, this conserved intracellular region is known to interact with G protein(s) (39). In the case of the α_1-adrenergic receptor, this conserved region BXBBXXB (B being a basic and X a nonbasic residue), which is highly homologous to that of the CCK_B-R (XBBBXXB), was shown to be specifically involved in PLC activation (40). In the light of our results, one can suppose that replacement of Asp^{100} by Asn modifies the conformation of the carboxyl terminal portion of the third intracellular loop of the receptor, thus affecting PLC activation and related intrinsic activity of the receptor mutant. Further site-directed mutagenesis experiments are under way in our laboratory to validate this hypothesis.

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