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The Third Intracellular Loop of the Rat and Mouse Cholecystokinin-A Receptors Is Responsible for Different Patterns of Gene Activation

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ABSTRACT

It has previously been reported that the cholecystokinin analog JMV-180 behaves differently on the rat and the mouse cholecystokinin-A receptor (CCK-AR). In mice this analog acts as an agonist on low- and high-affinity sites of the CCK-AR, whereas in rats this compound acts as an agonist on high-affinity sites and as an antagonist on low-affinity sites. In an attempt to understand why the same compound behaves differently on these two CCK-A receptors, we cloned the cDNA encoding the mouse CCK-AR. We then investigated a cellular model able to mimic the effect that was observed in rats and mice. HeLa cells were transiently cotransfected with plasmids leading to expression of the rat or mouse CCK-AR in the presence of pFos-Luc as reporter plasmid; such a plasmid placed the regulatory part of the human *c-Fos* gene upstream from the firefly luciferase structural gene (*Luc*). We then observed that the two CCK-A receptors behaved differently, not only in the presence of compound JMV-180 but also in the presence of cholecystokinin or even in absence of ligand; the rat CCK-AR was 2 to 3 times more potent than the mouse CCK-AR in inducing the reporter protein, whatever the ligand studied. This result was confirmed using the same kind of experiment with the reporter plasmid $p(TRE)_3$-tk-Luc. Using various mutated receptors, we investigated the role of the putative third intracellular loop. We concluded that both the primary structure of the receptor and the cellular context are in part responsible for the differential behavior of these CCK-A receptors.

Cholecystokinin (CCK) plays a major role as a hormone and neuropeptide, peripherally in the gastrointestinal system and centrally in the nervous system. Events of physiological and clinical importance are initiated by binding of CCK to its receptors. Receptors for CCK have been classified biologically and pharmacologically into two main types based on their relative affinity for sulfated or unsulfated CCK and on selective antagonist binding. The CCK-A receptor (CCK-AR) binds sulfated CCK-8 with a maximum affinity of 20 pM but has greater than 1000-fold lower affinity for unsulfated CCK-8 or gastrin-17. In contrast, the gastrin/CCK-B receptor (CCK-BR) binds CCK-8 and gastrin-17 with similar nM affinity and does not require ligand sulfation for binding (Shulkes and Baldwin, 1997). CCK-A receptors are found mainly in the peripheral system and to a lesser extent in localized areas of the central nervous system, whereas CCK-BR has been localized mainly throughout the central nervous system (Williams and Blevins, 1993; Wank, 1995; Shulkes and Baldwin, 1997). CCK-AR is involved in biological functions such as stimulation of digestive enzyme secretion, pancreatic growth, pathogenesis of schizophrenia, Parkinson's disease, drug addiction, and feeding disorders. CCK-AR and CCK-BR belong to the family of the G protein-coupled receptors and act by induction of intracellular messengers, primarily inositol triphosphate and diacylglycerol (Schulz, 1989; Williams et al., 1989; Williams and Blevins, 1993; Williams and Yule, 1993; Wank, 1995; Shulkes and Baldwin, 1997).

Stimulation of CCK-AR in rat pancreatic acini by CCK-8 results in a biphasic dose-response curve of amylase release (Peikin et al., 1978; Williams et al., 1978; Pandol et al., 1985; Bruzzone et al., 1986). Indeed, amylase release first increases at low CCK-8 concentrations (from 1 to 100 pM),

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ABBREVIATIONS: CCK, cholecystokinin; CCK-AR, cholecystokinin-A receptor; CCK-BR, cholecystokinin-B receptor; CCK-8, H-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2; BH-CCK-8, Bolton-Hunter cholecystokinin (26–33) amide; JMV-180, butyloxycarbonyl-Tyr(SO_3H)-Ahx-Gly-Trp-Ahx-Asp_2-phenylethyl ester; Luc, luciferase; TRE, 12-*O*-tetradecanoylphorbol-13-acetate responsive element; MAP, mitogen-activated protein; SAP, stress-activated protein; PCR, polymerase chain reaction.

whereas it decreases at higher CCK-8 concentrations (from 100 pM to 10 nM). Because there are two classes of sites for binding of CCK-8 to CCK-AR, it has been proposed that the upstroke of the dose-response curve for amylase release reflects occupation of the high-affinity binding sites of CCK-AR, and the downstroke of the dose-response curve reflects occupation of the low-affinity binding sites. These findings were supported both by binding experiments and by analysis of correlation of CCK-AR occupancy. It was further suggested that the high-affinity sites of CCK-AR stimulate amylase release, whereas the low-affinity sites of CCK-AR inhibit amylase secretion (Sankaran et al., 1980, 1982; Stark et al., 1989; Wank, 1995; Shulkes and Baldwin, 1997).

Evidence exists showing that JMV-180, a CCK-8 analog, induces pharmacological effects and signal transduction pathways distinct from those of the natural agonist CCK-8 through binding to CCK-AR. Moreover, the actions of JMV-180 can differ as a function of the animal species studied. For instance, in rat pancreatic acini, a model studied extensively, the dose-response curve for amylase secretion stimulated by JMV-180 is monophasic in that, unlike stimulation by CCK-8, no inhibitory phase is observed for supramaximal concentrations of the peptide analog (Lin et al., 1986; Galas et al., 1988; Matozaki et al., 1989; Stark et al., 1989). In contrast, in mouse pancreatic acini, the dose-response curve for amylase secretion stimulated by JMV-180 resembles that obtained with CCK-8. It has been suggested that JMV-180 acts as an agonist on the low-affinity sites of the mouse CCK-AR, whereas it acts as an antagonist on the low-affinity sites of the rat CCK-AR (Matozaki et al., 1989; Bianchi et al., 1994).

In the present study, we addressed the question of the differential response of CCK-AR from mice and rats to JMV-180. We cloned the mouse CCK-AR and studied the biological responses induced by this receptor in comparison with the rat CCK-AR in transfected COS-7 and HeLa cells. When looking at biological responses relatively near the cellular membrane (e.g., inositol phosphate hydrolysis), we were not able to discriminate between mouse and rat CCK-A receptors. This suggests that their differential behavior could be the result of differences in the cellular context of mouse and rat pancreatic acini. When looking at biological responses downstream of the membrane (e.g., activation of the c-Fos promoter), we could discriminate between the two CCK-A receptors. With the help of additional experiments using mutated forms of these receptors, we demonstrated that the primary structure of the receptor also contributes to the differential behavior of these two receptors.

Experimental Procedures

Materials. The origin of the compounds has been described previously (Oiry et al., 1997). ^{125}I-Bolton Hunter reagent (2000 Ci/mmol) and myo-[2-^{3}H]inositol (10–20 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Saclay, France). The reporter plasmid pFos-Luc was a gift from Dr. J. Tavare and Dr. M. Griffith (University of Bristol, Bristol, UK); this plasmid carries the luciferase structural gene (pGL3; Promega, Charbonnières, France) downstream from the human c-Fos promoter. The reporter plasmid p(TRE)$_3$-tk-Luc was a gift from Dr. M. Pons (INSERM U439, Montpellier, France); this plasmid expresses the firefly luciferase under the control of three repeated activator protein-1 regulatory sequences (TGAGTCA) (Astruc et al., 1995). The plasmid leading to the rat CCK-AR expression was prepared as described previously (Wank et al., 1992).

Cell Culture. HeLa and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, glutamine (2 mM), and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) as described previously (Oiry et al., 1997).

Cloning of the cDNA Encoding the Mouse CCK-AR. By using mouse pancreas poly(A)$^+$ RNA (CLONTECH/Ozyme, Bretonneux, France), first-strand synthesis and then second-strand synthesis were realized according to the manufacturer's recommended protocol (kit great lengths cDNA synthesis; Clontech). Double-stranded cDNA served as template for polymerase chain reaction (PCR) with 0.4 μM oligonucleotide sequences P1 (5′-GGCCGAATTCCCACCATGGATGTGGTCGACAGCC TT-3′) and P2 (5′-TCGATCTAGATCAGGGGGGTGGAGCAGAGGT-3′) (Oligo Express, Paris, France). These primers contained the sequences that are localized at the 5′- and 3′-ends of the published coding sequence of the rat CCK-AR (Wank et al., 1992); in addition, the primer P1 contained the initiation codon ATG, which was inserted into the Kozak consensus sequence for favored initiation of translation (Kozak, 1987). These primers also contained restriction sites for *Eco*RI (in primer P1) and for *Xba*I (in primer P2). PCR was performed with a MiniCycler (MJ Research, Prolabo, France) in a 50-μl solution containing 5 μl of 10× *Pfu* buffer, 0.5 mM deoxyribonucleotide triphosphates, 0.4 μM primers, 1–5 ng of cDNA sample, and 2.5 U of recombinant *Pfu* polymerase (CLONTECH). The following cycle temperatures and times were used under standard PCR conditions: denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 3 min, 40 cycles, final extension at 72°C for 10 min. The products from different PCRs that were pure were sent to Genome Express (Meylan, France) for sequencing. At the same time, the PCR product (*Eco*RI-*Xba*I piece) was subcloned into the vector pCiNeo (Promega). The resulting construct called p(mouse/CCK-AR/pCiNeo) was amplified in competent cells (*Escherichia coli* JM109, Promega). Classical methods were applied for biological molecular protocols (Sambrook et al., 1989).

Mutants. The p(mouse/CCK-AR/pCiNeo) was mutated to lead to the expression of two mutants of the mouse CCK-AR: S1 and T15. S1 corresponds to the mouse CCK-AR, in which the third intracellular loop of the mouse CCK-AR has been totally replaced by that of the rat CCK-AR. T15 corresponds to the mouse CCK-AR, in which amino acids 294 to 308 of the third intracellular of the mouse CCK-AR have been replaced by those of the rat CCK-AR. The protocol followed has been described previously (Gigoux et al., 1998).

Transient Transfection. All plasmids used in transfection experiments were purified according to the alkaline lysis method by using the Plasmid Maxi Kit (Qiagen, Courtaboeuf, France). For binding experiments and for total inositol phosphate assays, COS-7 cells were plotted in 100-mm Petri dishes (2×10^6 cells/plate). One day later, cells were transfected with the plasmid leading to expression of the mouse or the rat CCK-AR (2–4 μg/plate) according to the DEAE-dextran method as described previously (Sambrook et al., 1989; Gigoux et al., 1998). For reporter gene analysis, HeLa and COS-7 cells were plotted as above before cotransfection by the calcium phosphate method with the reporter plasmid [pFos-Luc or p(TRE)$_3$-tk-Luc, ca. 5 μg/dish] and with the plasmid leading to expression of the mouse (wild-type or mutant S1 or T15) or the rat CCK-AR (ca. 5 μg/dish) (Sambrook et al., 1989; Astruc et al., 1995; Oiry et al., 1997).

Receptor Binding Experiments. One day after the transfection, cells were trypsinized and plotted in 12- to 24-well tissue culture cluster plates, and 24 h later cells were treated as described previously for binding experiments (Gigoux et al., 1998). Results are expressed as means of percentages of specific binding ± S.D. by taking as 100% the radioactivity specifically bound at 0.05 nM ^{125}I-BH-CCK-8. The nonspecific binding was determined by the radioactivity measured in the presence of 0.05 nM ^{125}I-BH-CCK-8 and a 500-fold excess of cold CCK-8. Nonspecific binding was always less

than 10% of the total binding. As indicated, such experiments were performed to study the ability of several compounds to displace ^{125}I-BH-CCK-8 binding.

Total Inositol Phosphate Assays. This was performed on acini that were isolated from rat and mouse pancreas as described previously (Matozaki et al., 1989). Pancreatic acini were loaded with 1 to 2 μM myo-[2-^{3}H]inositol (10–20 Ci/mmol) for 2 h at 37°C. After this incubation, acini were extensively washed with PBS or culture medium 199 and then treated for 15 min at 37°C with 10 mM LiCl. Pancreatic acini were then incubated with different concentrations of CCK-8 or JMV-180 for 30 min at 37°C (for each experimental point, the final volume was 0.5 ml). Incubation was then stopped at 4°C by adding 0.5 ml of $HClO_4$ (5%, v/v); each tube then received 2 M K_2CO_3 (0.155 ml) and 0.4 M HEPES (0.025 ml). The tubes were centrifuged (3000 rpm, 10 min). A 0.9-ml aliquot fraction of the supernatant was taken before washing the pellet with 0.5 ml of H_2O. After centrifugation, a 0.5-ml aliquot fraction was pooled with the previous 0.9 ml. The mixture was then applied to Dowex AG 1-X8 resin [200- to 400-mesh formate form (Bio-Rad, Marnes-la-Coquette, France)] to determine total inositol phosphate contents by gel chromatography (Gigoux et al., 1998). Columns were washed with water and then with 40 mM ammonium formate, and elution was finally performed with 1 M ammonium formate. The eluates were then assayed for their radioactivity contents. The same kind of experiments was also performed in COS-7 and HeLa cells expressing the mouse or the rat CCK-AR. The protocol was similar to that described above, except that 1 day after transfection, cells were incubated for 24 h at 37°C with 1 to 2 μM myo-[2-^{3}H]inositol (10–20 Ci/mmol) in medium 199. Cells were then incubated with 10 mM LiCl for 15 min at 37°C before realizing a cellular suspension to obtain ca. 0.5 to 1 $\times$ 10^6 cells/experimental point. The experiment was then followed as above; for both experiments, results are expressed as mean percentages $\pm$ S.D. by taking as 100% the maximum level of total inositol phosphate induced by CCK-8.

Luciferase Assays. One day after the transfection, cells were trypsinized and plotted in 12- to 24-well tissue culture cluster plates. Once attached to the support, cells were extensively washed before culture in medium supplemented with 0.3% fetal calf serum. Twenty-four hours later, cells were incubated with the tested compounds. After incubation for 8 h, cells were treated as described previously for assaying the luciferase activity (Astruc et al., 1995). Results are expressed as indicated (mean arbitrary units $\pm$ S.D.).

Results

Cloning of the cDNA Encoding the Mouse CCK-AR. The cDNA encoding the mouse CCK-AR was obtained after first- and second-strand synthesis of mRNAs from mouse pancreas. This cDNA was then amplified by PCR as indicated under *Experimental Procedures*. Several groups have been investigating the cloning and sequencing of the cDNA and/or of the gene of the mouse CCK-AR (Ghanekar et al., 1997; Lacourse et al., 1997; Takata et al., 1997). Our results are in full agreement with these studies and indicate a high degree of homology between the amino acid sequences of the mouse and rat CCK-AR receptors. Table 1 summarizes the differences: two differences appear in the first putative transmembrane domain (L_{43} and I_{50} in the rat CCK-AR are replaced by V_{43} and F_{50}, respectively, in the mouse CCK-AR); two differences appear in the fifth putative transmembrane domain (L_{220} and I_{223} in the rat CCK-AR are replaced by I_{220} and V_{223}, respectively, in the mouse CCK-AR). The major differences appear in the third putative intracellular loop because a box containing a GGGGGGS sequence is present in the mouse CCK-AR at positions 259 to 265, whereas this box is absent in the rat CCK-AR. Moreover, K_{254}, P_{255}, T_{257}, T_{261}, S_{287}, G_{288}, G_{290}, S_{292}, L_{294}, and S_{301} in the rat CCK-AR are replaced, respectively, by R_{254}, L_{255}, S_{257}, S_{268}, T_{294}, S_{295}, S_{297}, G_{299}, I_{301}, and G_{308} in the mouse CCK-AR. Finally, two differences appear in the C-terminal part (P_{395} and L_{413} in the rat CCK-AR are replaced by T_{402} and S_{420}, respectively, in the mouse CCK-AR).

Functional Characteristics of the Mouse CCK-AR (Binding Experiments). The cDNA cloned from mouse pancreas and that encoding the rat CCK-AR were transiently transfected in COS-7 cells for pharmacological and functional analyses. Both receptors bound the CCK-8 radioligand with a high affinity. Maximal binding capacities of both CCK-AR variants were similar, demonstrating similar levels of expression (for six different experiments, the B_{max} values corresponding to cells expressing the mouse or rat CCK-AR were 13.04 $\pm$ 1.82 and 9.66 fmol/10^6 cells $\pm$ 1.5, respectively). The pharmacological properties of these two receptor variants, summarized in Table 2, demonstrate that the rat and mouse CCK-AR display indistinguishable pharmacological features toward all the tested compounds. The IC_{50} values of the different compounds were 1 nM for CCK-8 and 20 to 30 nM for the different CCK-8 receptor antagonists. The IC_{50} value of JMV-180 was 20 nM for the rat CCK-AR and 30 nM for the mouse CCK-AR. Two ligands known to specifically bind the CCK-BR (L-365,260 and PD-135,158) were unable to bind the mouse or rat CCK-AR. All these results are in full agreement with several data obtained in several rat and mouse models (Matozaki et al., 1989; Stark et al., 1989; Wank, 1995).

Investigation of a Cellular Model to Study the Differential Behavior of JMV-180 on the Rat and Mouse CCK-AR. We have investigated the effects of compound JMV-180 on total inositol phosphate production in COS-7 cells expressing the rat CCK-AR or the mouse CCK-AR. Indeed, although CCK-AR activation by CCK-8 is known to

TABLE 1

Differences in amino acids between rat and mouse CCK-AR

The place of each different amino acid is indicated by referring to its place in the mouse CCK-AR; its localization in the receptor is also indicated (TM1, putative first transmembrane domain; TM 5, putative fifth transmembrane domain; i3, putative third intracellular loop; Cter, C-terminal part).

Amino Acid No.	Rat CCK-AR	Mouse CCK-AR	Putative Domain	Amino Acid No.	Rat CCK-AR	Mouse CCK-AR	Putative Domain
43	L	V	TM1	294	S	T	i3
50	I	F	TM1	295	G	S	i3
220	L	I	TM5	297	G	S	i3
223	I	V	TM5	299	S	G	i3
254	K	R	i3	301	L	I	i3
255	P	L	i3	308	S	G	i3
257	T	S	i3	402	P	T	Cter
259–265		[GGGGGGS]	i3	420	L	S	Cter
268	T	S	i3				

lead to activation of phospholipase C-β in rat pancreatic acini and in transfected cells (Berridge and Irvine, 1989; Rowley et al., 1990; Yule et al., 1993; Gaisano et al., 1994), the effect of JMV-180 on inositol phosphate production is relatively controversial (from 0 to 28% of the maximum level produced by CCK-8) (Rowley et al., 1990; Yule et al., 1993; Gaisano et al., 1994). On the other hand, the coupling of the CCK-AR to inositol phosphate production in mouse pancreatic acini was poorly studied. As shown in Fig. 1A, in both rat and mouse pancreatic acini, CCK-8 induced inositol phosphate production in a dose-dependent manner with an EC_{50} value near 1 nM and with an induction ratio of 5-fold over the basal value. In contrast, compound JMV-180 stimulated inositol phosphate production in mouse pancreatic acini but was ineffective in rat pancreatic acini. In mouse pancreatic acini, JMV-180 induced inositol phosphate production in a dose-dependent manner but with less efficacy than CCK-8, because 10 μM JMV-180 gave 40% of the maximal response obtained with 100 nM CCK-8.

A similar study was performed in COS-7 cells that were transiently transfected with the plasmid encoding the rat or mouse CCK-AR and then stimulated by CCK-8 and JMV-180. Figure 1B shows that, as in pancreatic acini, CCK-8 was able to induce inositol phosphate production in a dose-dependent manner with an EC_{50} value near 1 nM and an induction ratio of 6 for the two CCK-AR variants. Contrary to that observed in pancreatic acini (Fig. 1A), compound JMV-180 was able to induce inositol phosphate production in COS-7 cells expressing the mouse or rat CCK-AR. In both cases, the results showed a similar pattern because 1 μM JMV-180 produced 20 to 40% of the maximal response obtained with 100 nM CCK-8.

The weak differences that we observed between rat and mouse CCK-AR on inositol phosphate production in COS-7 cells stimulated by JMV-180 seemed statistically insignificant. This suggested that the differential behavior of JMV-180 in rat and mouse species could reside, at least in part, in species specificities of acinar cells.

The relation among inositol phosphate production, intracellular Ca^{2+} mobilization, and amylase secretion was analyzed. Because intracellular Ca^{2+} mobilization and biological effects could be observed while inositol phosphates were not detected, it was suggested that an amplification step could exist between inositol phosphate production and biological events such as intracellular Ca^{2+} mobilization and amylase secretion (Rowley et al., 1990).

A cellular model able to better characterize the differential behavior of JMV-180 toward the rat and mouse CCK-AR was thus investigated by taking a biological response downstream inositol phosphate production. We used the reporter gene strategy with a promoter known to be activated by multiple factors, including G protein-coupled receptors. We choose the c-Fos promoter because this promoter is the target of several complex kinase pathways activated by events initiated at the membrane level (Janknecht et al., 1995; Treisman, 1995; Gutkind, 1998). The choice of this promoter was based on the fact that, in the rat model (rat pancreatic acini, AR42J), activation of the CCK-AR by CCK-8 increased c-Fos mRNA and c-Fos protein expression. In these studies compound JMV-180 was shown to behave as a partial agonist (Muller et al., 1984; Lu and Logsdon, 1992; Logsdon et al., 1994). COS-7 and HeLa cells were transiently cotransfected with the plasmid encoding the rat or mouse CCK-AR in the presence of the reporter plasmid pFos-Luc. Figure 2 shows that luciferase inducibility seemed to follow two relatively different profiles, depending on which CCK-AR (mouse or rat) was expressed. In cells expressing the rat CCK-AR, the response profile was more extended than that obtained in cells expressing the mouse CCK-AR. This was observed for all compounds tested (CCK-8 and JMV-180) but also in the absence of any ligand (for each expressed CCK-AR, the effects of control, JMV-180 and CCK-8 on luciferase activity defined the response profile of the given CCK-AR). Such a differential behavior between these two receptors could not reside in transfection variations because these receptors were expressed at similar levels (as indicated above, the B_{max} values corresponding to cells expressing the mouse or rat CCK-AR were 13.04/10^6 cells $\pm$ 1.82 and 9.66 fmol/10^6 cells $\pm$ 1.5, respectively); moreover, by transfection of increasing quantities of plasmids encoding the rat or mouse CCK-AR (plasmid quantity ranging from 0.1 to 1 μg/10^6 cells), their response profiles were maintained differently (not shown). For both rat and mouse CCK-A receptors, CCK-8 induced luciferase activity in a dose-dependent manner with an EC_{50} value of 0.3 to 1 nM. Under these experimental conditions, the induction ratio of 30 nM CCK-8 was 2.5 $\pm$ 0.28 in cells expressing the mouse CCK-AR, whereas it was 4.1 $\pm$ 0.3 in cells expressing the rat CCK-AR. The induction ratio of 1 μM JMV-180 was 2.04 $\pm$ 0.17 in cells expressing the mouse CCK-AR (corresponding to an efficacy of 70–80% of the maximal response obtained with 30 nM CCK-8), whereas it was 2.13 $\pm$ 0.22 in cells expressing the rat CCK-AR (30–40% of the maximal response obtained with 30 nM CCK-8). In the same type of experiments, compound JMV-180 was tested for its ability to antagonize the CCK-8 effect on luciferase activity. JMV-180 caused a 20 to 30% inhibition of the CCK-8 effect in cells expressing the mouse CCK-AR and a 60 to 70% inhibition in cells expressing the rat CCK-AR (not shown).

To confirm the differential behavior of these two receptors, we transiently cotransfected HeLa cells with plasmids encoding the rat or mouse CCK-AR in presence of another reporter gene whose activity depended at least in part on c-Fos protein level. This was investigated using p$(TRE)_3$-tk-Luc as reporter plasmid (Astruc et al., 1995). The TRE sequence has been described extensively as a binding site for activator protein-1, comprising protein dimers containing the gene

TABLE 2

Ability of various ligands to inhibit binding of ^{125}I-BH-CCK-8 in COS-7 cells transiently expressing the rat or mouse CCK-AR

The IC_{50} values were determined as indicated under *Experimental Procedures* and in the text. Each value represents the mean $\pm$ S.D. of at least three separate experiments performed in triplicate. For each experiment, the nonspecific binding was approximately 5 to 10% of the total binding.

Ligand	Mouse CCKAR K_i	Rat CCKAR K_i
	nM	
CCK-8	1 $\pm$ 0.3	1 $\pm$ 0.3
JMV-180	30 $\pm$ 2.5	20 $\pm$ 2.5
SR-49,344	25 $\pm$ 2	20 $\pm$ 2.5
L-364,718	25 $\pm$ 2.5	12.5 $\pm$ 2.5
JMV-379	35 $\pm$ 2.5	35 $\pm$ 2.5
PD-170,292	35 $\pm$ 2.5	25 $\pm$ 2.5
L-365,260	nd	nd
PD-135,158	nd	nd

nd, no displacement.

products of members of the *jun* and *fos* gene families. As shown in Fig. 3, both receptors behaved in an identical manner whether the cells were transfected with the reporter plasmid pFos-Luc (Fig. 3A) or p(TRE)$_3$-tk-Luc (Fig. 3B). Again, the CCK-8-induced luciferase seemed to follow two profiles relatively differently, depending on the rat or mouse CCK-AR which is expressed. The response profile obtained in the presence of the rat CCK-AR was more extended than the profile obtained in the presence of the mouse CCK-AR. Again, the differential behavior of these receptors was observed for both compounds tested (CCK-8 and JMV-180) and also in the absence of any ligand. In experiments using p(TRE)$_3$-tk-Luc as reporter plasmid (Fig. 3B), the induction ratio of 30 nM CCK-8 was 4.03 ± 0.4 in cells expressing the mouse CCK-AR, whereas it was 4.83 ± 0.15 in cells expressing the rat CCK-AR; the induction ratio of 1 μM JMV-180 was 3.08 ± 0.19 in cells expressing the mouse CCK-AR (corresponding to an efficacy of 70–80% of the maximal response obtained with 30 nM CCK-8), whereas it was 2.73 ± 0.1 in cells expressing the rat CCK-AR (30–40% of the maximal response obtained with 30 nM CCK-8). On such cellular models that distinguished the mouse CCK-AR from the rat CCK-AR, we decided to investigate the role of the putative third intracellular loop of these receptors. Two mutants of the mouse CCK-AR cDNA were thus prepared as indicated under *Experimental Procedures*. S1 corresponded to the mouse CCK-AR in which the putative third intracellular loop is that of the rat CCK-AR, and T15 corresponded to the mouse CCK-AR in which the putative third intracellular loop is that of the rat CCK-AR for amino acids 294 to 308. Using experiments of the same kind as described above, the behavior of these two mutants was investigated. As shown in Fig. 4, whatever the reporter plasmid, pFos-Luc (Fig. 4A) or p(TRE)$_3$-tk-Luc (Fig. 4B), the same profiles of results were obtained. For both reporter plasmids, the effects of JMV-180 and of CCK-8, as well as that of the vehicle, clearly showed that the mutant S1 behaved like the rat CCK-AR, whereas the mutant T15 behaved like the mouse CCK-AR. Again, these different response profiles could not be attributed to different levels of expression (the B_{max} values corresponding to cells expressing mouse or rat CCK-AR and the S1 or T15 mutant forms of CCK-AR were 13.04 ± 1.82, 9.66 ± 1.5, 13.2 ± 2.24, and 13.3 fmol/10^6 cells ± 2.16, respectively). Under our experimental conditions, the results obtained with mutated forms of CCK-AR clearly indicated that differences in the primary structure of the putative third intracellular loop of these receptors are responsible for their differential behavior on *c-Fos* gene activation and probably on other gene(s) for which expression is dependent on the c-Fos protein level.

Discussion

JMV-180 (a CCK-8 analog) is an agonist for both high- and low-affinity sites of the mouse CCK-AR and an agonist for high-affinity sites and antagonist for low-affinity sites of the rat CCK-AR. Structural and biochemical parameters responsible for this specific behavior of JMV-180 at low-affinity binding sites of the CCK-AR are crucial to control. They can potentially provide significant information for the elaboration of new compounds particularly useful in obesity treatment (Asin and Bednarz, 1992). To address this question, we first cloned and sequenced the cDNA encoding the mouse CCK-AR. The deduced amino acid sequences revealed differences compared with those of the rat: in the putative first transmembrane domain by two amino acids, in the putative fifth transmembrane domain by two amino acids, in the putative third intracellular loop by 17 amino acids, [including a (GGGGGGS) box that is absent in the rat CCK-AR], and in the putative C-terminal part by two amino acids (see Table 1). We performed a pharmacological study of this cloned cDNA expressed in COS-7 cells by testing several molecules known to interact with the CCK-AR. No significant differences were observed in binding experiments for the rat or mouse CCK-AR. We then investigated a cellular model able to mimic the differential behavior of JMV-180 toward the rat or mouse CCK-AR. Because this kind of receptor has been described as related to phospholipase C-β activation (Ber-

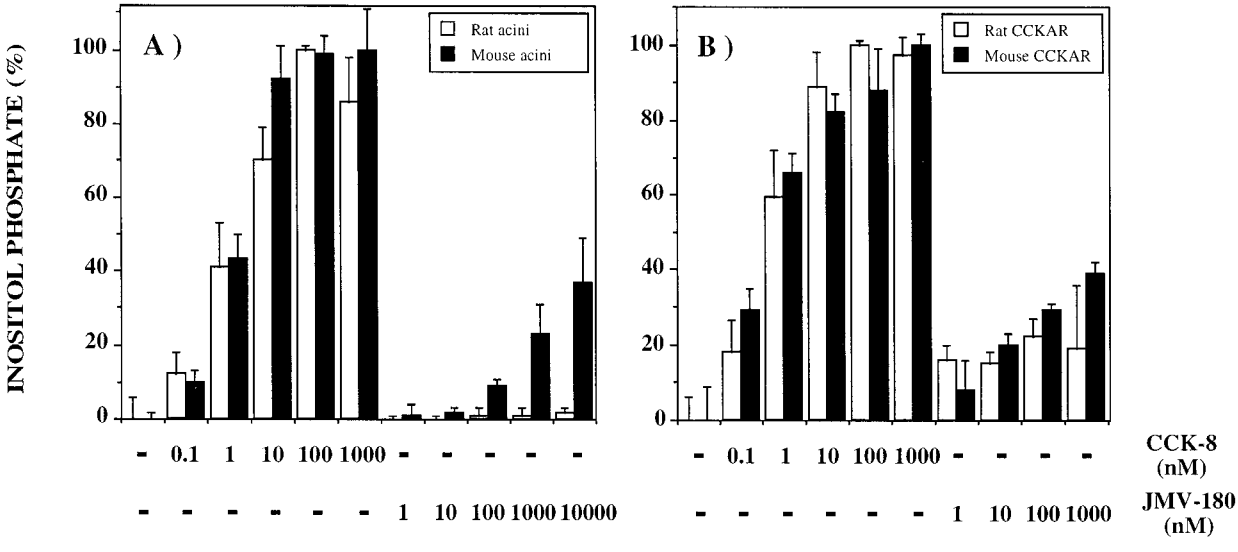

Fig. 1. Effect of JMV-180 and of CCK-8 on total inositol phosphate production in pancreatic acini from the rat or mouse (A) and in COS-7 cells transiently transfected with plasmid expressing rat or mouse CCK-AR (B). Pancreatic acini and COS-7 cells were treated as described under *Experimental Procedures* for total inositol phosphate quantification. Results are expressed as percentages, where 100% is the maximal inositol phosphate production induced by CCK-8. Each value represents the mean ± S.D. of at least three separate experiments performed in duplicate.

ridge and Irvine, 1989; Williams and Blevins, 1993; Wank, 1995; Shulkes and Baldwin, 1997), COS-7 cells were transiently transfected with plasmids encoding the rat or the mouse CCK-AR, and then inositol phosphate production was measured after cells were treated with JMV-180 or with CCK-8. JMV-180 had the same partial agonist activity in cells expressing the rat CCK-AR or the mouse CCK-AR (about 20 and 40%, respectively, of the maximum level produced by CCK-8). In contrast, on mouse pancreatic acini, JMV-180 exhibited a partial agonist activity of 40% of the maximum response induced by CCK-8, whereas in rat pancreatic acini, no agonist activity at all could be detected.

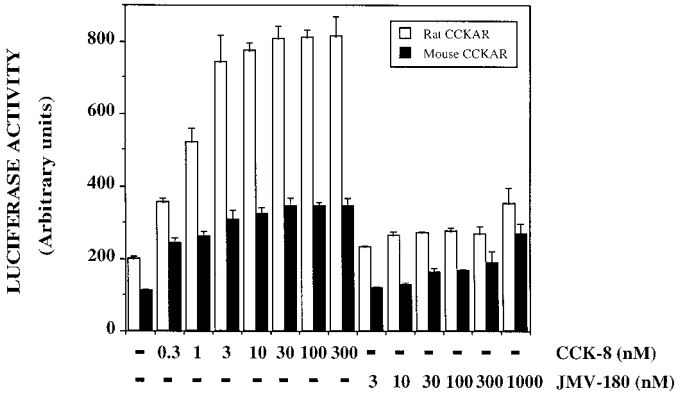

Fig. 2. Effect of JMV-180 and of CCK-8 on luciferase activity resulting from activation at the human c-Fos promoter in HeLa cells expressing the rat or mouse CCK-AR. As indicated under *Experimental Procedures*, HeLa cells were transiently cotransfected with plasmids leading to expression of the indicated CCK-AR and pFos-Luc as reporter plasmid. Results are expressed in arbitrary units [luminescence value indicated by the luminometer at which the background noise (a value of approximately 8–10) was substracted]. Each value represents the mean ± S.D. of at least three separate experiments performed in triplicate. As indicated in the text, when cells expressed the rat CCK-AR, the induction ratio was 2.13 ± 0.22 in the presence of 1 μM JMV-180 and 4.1 ± 0.3 in the presence of 30 nM CCK-8. When cells expressed the mouse CCK-AR, the induction ratio was 2.04 ± 0.17 in the presence of 1 μM JMV-180 and 2.5 ± 0.28 in the presence of 30 nM CCK-8.

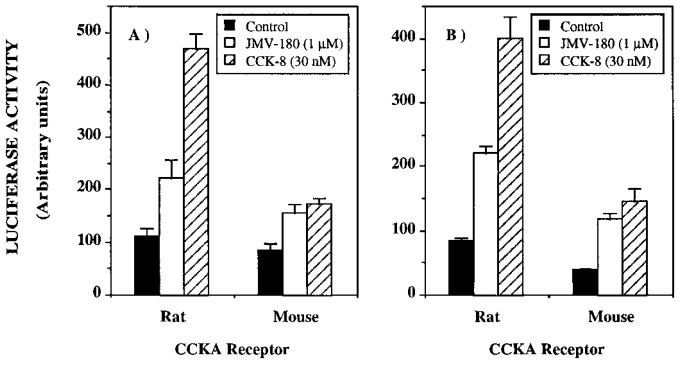

Fig. 3. Effect of JMV-180 and of CCK-8 on luciferase activity resulting from activation at the human c-Fos promoter (A) or at a TRE sequence (B) in HeLa cells expressing the rat or mouse CCK-AR. As indicated under *Experimental Procedures*, HeLa cells were transiently cotransfected with plasmids leading to expression of the indicated CCK-AR and pFos-Luc (A) or p(TRE)$_3$-tk-Luc (B) as reporter plasmid. Results are expressed as indicated in the legend to Fig. 2. The various induction ratios indicated in the legend to Fig. 2 are very near those obtained in the experiments shown in Fig. 2A. In the experiments shown in Fig. 2B, when cells expressed the rat CCK-AR, the induction ratio was 2.73 ± 0.1 in the presence of 1 μM JMV-180 and 4.83 ± 0.15 in the presence of 30 nM CCK-8, whereas when cells expressed the mouse CCK-AR, the induction ratio was 3.08 ± 0.19 in the presence of 1 μM JMV-180 and 4.03 ± 0.4 in the presence of 30 nM CCK-8.

Results obtained with JMV-180 in COS-7 cells expressing the rat CCK-AR are very different from those obtained in rat pancreatic acini. However, in COS-7 cells expressing the mouse CCK-AR, JMV-180 behaved as in mouse pancreatic acini. This suggested that the differential behavior of JMV-180 for rat or mouse CCK-AR on amylase secretion could reside in a different cellular context related to species differences. Our conclusion is supported by Ghanekar et al. (1997), who suggested that differential coupling to G protein may depend on the cellular context; for example, this may relate to differences extrinsic to the CCK-A receptor in the stoichiometry or character of G proteins or in the composition or organization of the lipid environment of the acinar cell membrane. Our results are also in agreement with other studies showing that JMV-180 is relatively ineffective in stimulating inositol phosphate production compared with CCK-8 both in rat pancreatic acini and in Chinese hamster ovary cells stably transfected with the cDNA encoding the rat CCK-AR (Rowley et al., 1990; Yule et al., 1993; Gaisano et al., 1994). In the same rat models, JMV-180 or its analog (2-phenylethylester) was shown to be a partial agonist for stimulating the intracellular Ca^{2+} mobilization (approximately 50% of the maximum level produced by CCK-8), thus showing that inositol phosphate production could not be directly related to intracellular Ca^{2+} mobilization or to the biological effect. It was then suggested that an amplification step could exist between inositol phosphate production and biological events, such as intracellular Ca^{2+} mobilization and amylase secretion (Rowley et al., 1990). For this reason, we investigated a cellular model able to better characterize the differential behavior of JMV-180 toward the rat and mouse CCK-AR by studying a biological response downstream of inositol phosphate production. We chose the reporter gene strategy using the plasmid pFos-Luc in which the regulatory part of the human c-Fos promoter is placed upstream from the firefly luciferase structural gene (*Luc*). Using such a reporter, any induction of luciferase can be easily detected by bioluminescence and could be directly related to activation of the c-Fos promoter. The choice of this promoter resides in the fact that 1) c-Fos protein expression as well as c-Fos mRNA was shown to be increased in pancreatic acini following activation of the CCK-AR (Muller et al., 1984; Lu and Logsdon, 1992; Logsdon et al., 1994), 2) c-Fos induction involves low-affinity binding sites of the CCK-AR (Lu and Logsdon, 1992; Logsdon et al., 1994), and 3) the different responsive elements of the c-Fos promotor are targets of several kinase pathways (MAP kinases, SAP kinases/c-jun N-terminal kinase, SAP kinases/p38 MAP kinases), which are known to be activated by several membrane receptors including G protein-coupled receptors (Janknecht et al., 1995; Treisman, 1995; Gutkind, 1998). By transient cotransfection of COS-7 cells and HeLa cells with plasmids leading to expression of the mouse or rat CCK-AR in presence of pFos-Luc, we observed that luciferase inducibility followed two different profiles depending on the CCK-AR that is expressed. Luciferase activity was always 2 to 3 times higher when the rat CCK-AR was expressed than when the mouse CCK-AR was expressed, irrespective of the ligand tested: JMV-180, CCK-8, or vehicle. These results were confirmed using another reporter plasmid whose activation depends at least partly on the level of c-Fos protein (p(TRE)$_3$-tk-Luc).

As indicated above, the major differences between these

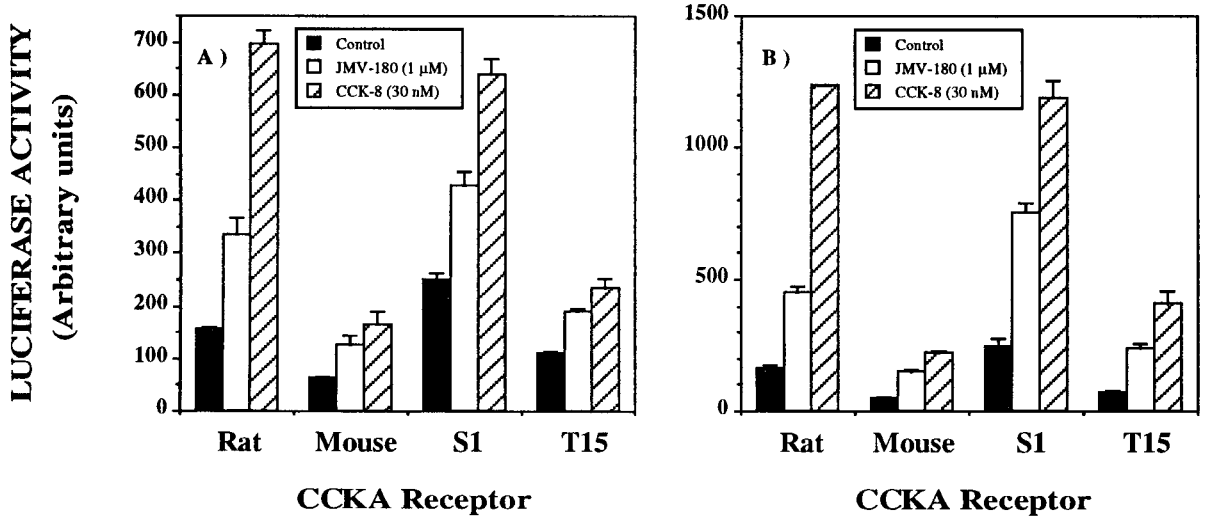

Fig. 4. Effect of JMV-180 and of CCK-8 on luciferase activity resulting from activation at the human c-Fos promoter (A) or at a TRE sequence (B) in HeLa cells expressing the rat or mouse CCK-AR or the S1 or T15 mutants of the mouse CCK-AR. As indicated under *Experimental Procedures*, HeLa cells were transiently cotransfected with plasmids leading to expression of the indicated CCK-AR and pFos-Luc (A) or p(TRE)$_3$-tk-Luc (B) as reporter plasmid. Results are expressed as indicated in the legend to Fig. 2. Each value represents the mean ± S.D. of a typical experiment repeated three times in triplicate.

two receptors reside in the putative third intracellular loop, a region that is very important in intracellular transduction of G protein-coupled receptors. We thus repeated the above experiments by testing two mutants of the mouse CCK-AR. Mutant S1 expressed a mouse CCK-AR in which the putative third intracellular loop was that of the rat CCK-AR. This mutant led to luciferase inducibility whose response profile corresponded to that of the rat CCK-AR. This clearly indicated that the putative third intracellular loop of the receptor is responsible for the difference in behavior between rat and mouse CCK-A receptors. Another mutant (T15) expressing the mouse CCK-AR, in which amino acids 294 to 308 of the putative third intracellular loop were replaced by those of the rat, led to luciferase inducibility whose response profile (vehicle, JMV-180 and CCK-8) corresponded to that of the mouse CCK-AR. This suggested that the N-terminal part of the putative third intracellular loop (namely, amino acids 254–268) is responsible for the differential behavior between the rat and mouse CCK-A receptors

We and others have clearly observed a differential behavior between mouse and rat CCK-A receptors in animals, whereas in transiently transfected cells such behavior has not been clearly observed. This could probably result from the distinct cellular context between animals and cultured cells. Logsdon (1999) also emphasized the influence of the cellular context on function and regulation of G protein-linked receptors (e.g., the M3 muscarinic acetylcholine, the gastrin releasing peptide, or the cholecystokinin A receptors). Activation of these receptors induces Ca^{2+} release from internal stores in various cell types, probably because these receptors have a high affinity for Gq subunits, which are ubiquitously expressed. In contrast, coupling to multiple signaling pathways may occur and could thus depend on several cellular parameters [e.g., promiscuous G protein coupling, separate effects of α- and $\beta\gamma$-subunits, signal "cross-talk," and activation of other signaling pathways (MAP kinases, SAP kinases/c-jun N-terminal kinase, SAP kinases/p38 MAP kinases)]. Different cells also respond differently to receptor activation. For example, activation of Gq-coupled receptors has been shown to lead to increased cellular growth rates in numerous cell types, whereas in other cell types, growth inhibition has been reported. In this case, the receptors may couple to different signals in the cells or cells may respond differently to the same signals depending on other aspects of the cell context. In addition, the cell context can affect receptor trafficking and desensitization, thus corresponding to a receptor loss or to an inaccessibility of the receptor by its ligand (Logsdon, 1999).

In this study, we demonstrated that the primary structure of the CCK-AR could also influence its behavior. This relies on the major observation that when the rat and mouse CCK-AR are "placed" in a same cellular context, they behaved differently, and this would depend on the primary structure of their putative third intracellular loop. In our hands, we could not discriminate between the two receptors when looking at a biological response very near the membrane (inositol phosphate production). On the contrary, we were able to differentiate them when looking at the nuclear level (gene activation). This would suggest that c-Fos induction is inositol phosphate independent; another alternative is that events relatively near the membrane do not take into account any amplification phenomena (Rowley et al., 1990).

Taken together, this suggests that parameters both extrinsic (cellular context) and also intrinsic to the CCK-AR (primary structure) could be responsible for the differential behavior of mouse and rat CCK-AR. This is also supported by a recent study (Ji et al., 2000).

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