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Mutational analysis of the putative devazepide binding site of the CCK_A receptor

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Abstract

Recently a molecular model was proposed for the binding site of the antagonist 3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-1*H*-indole-2-carboxamide (devazepide) on the cholecystokinin-A (CCK_A) receptor (Van der Bent et al., 1994. Drug Design Discov. 12, 129–148). Fifteen amino acids were identified, including hydrophilic ones such as Ser^{139}, Asn^{349} and Ser^{379}, that might interact with the carboxamide moiety in devazepide. To provide mutational evidence for this model, wild-type and mutant receptors (S139A, N349A and S379A) were transiently expressed and compared with respect to the ability of devazepide to inhibit binding of radiolabelled cholecystokinin-(26–33)-peptide amide (CCK-8) and CCK-8-evoked Ca^{2+} mobilization. The data presented suggest the involvement of the three residues in antagonist binding, although to a different extent. However, it does not seem likely that hydrogen bonds are the driving force in view of the relatively minor changes in receptor affinity and activity. © 1997 Elsevier Science B.V.

Keywords: CHO (Chinese hamster ovary) cell; Cholecystokinin; CCK_A receptor; Devazepide; L-364,718; Ca^{2+} mobilization; Cell recruitment; Receptor modelling

1. Introduction

Receptors interacting with the cholecystokinin (CCK) and gastrin families of peptides are widely expressed throughout the gastrointestinal and nervous system (Wank, 1995). Recent primary amino acid sequence deduction of various CCK receptor cDNAs has strengthened previous pharmacological evidence for the existence of two receptor subtypes, designated CCK_A and CCK_B, the latter being identical to the gastrin receptor. The CCK_A receptor predominates in the gastrointestinal tract, whereas the CCK_B receptor is predominantly distributed throughout the brain and spinal cord. The extensive regulatory role of CCK in the gastrointestinal tract has stimulated the interest in developing receptor ligands with (ant)agonistic modes of action for the treatment of several gastrointestinal disorders (Wank, 1995). Thus far, two CCK_A receptor antagonists, loxiglumide (Meyer et al., 1989) and devazepide (3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-1*H*-indole-2-carboxamide; Liddle et al., 1989), have entered clinical studies. However, caution should be taken since animal toxicity studies revealed that long-term treatment with devazepide may lead to serious side effects (Iversen et al., 1991).

In order to provide a more rational approach towards the design of receptor antagonists, Van der Bent et al. (1994) previously built a bacteriorhodopsin-based model of the membrane-embedded part of the CCK_A receptor in which they identified a putative antagonist binding site on the basis of the structure-activity relationships and conformational characteristics of the two parent classes of CCK_A receptor antagonists, lorglumide and devazepide. The most prominent feature of the proposed antagonist binding site is the presence of a hydrogen acceptor, the existence of which is concluded from the observation that the affinity of devazepide is highly dependent on the presence of a hydrogen atom on the exocyclic amide bond that connects the benzodiazepine and indole systems (Evans et al., 1988).

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Both replacement of this hydrogen by a methyl group and that of the entire amide bond by a methylene bridge results in an affinity loss of over 4 log units. Potential hydrogen acceptors are Ser^{139} (H3), Thr^{132} (H3), Asn^{349} (H6), Ser^{375} (H7) and Ser^{379} (H7). Of these, Ser^{139}, located nearly half-way helix 3 (H3) in the interior of the receptor pore, is the most likely candidate since attempts to dock devazepide with hydrogen bonds to Asn^{349}, Ser^{375} and Ser^{379} failed, whereas Thr^{132} was regarded as unlikely because of its location close to the extracellular end of the pore. Other amino acids, which, because of their location within 4 Å of the predicted docking site, form part of the putative antagonist binding site, are: Phe^{112}, Phe^{135}, Met^{136}, Ser^{143}, Trp^{181}, Ser^{184}, Leu^{232}, Trp^{342}, Ile^{345}, Leu^{372} and Tyr^{376}.

The present study aims to validate the proposed model for the antagonist binding site of the CCK_A receptor. To this end Ser^{139}, Ser^{379} and Asn^{349} were individually mutated into Ala. Mutated receptors were transiently expressed in Chinese hamster ovary (CHO-K1) cells and the potency of devazepide to inhibit the binding of radiolabelled cholecystokinin-(26–33)-peptide amide (CCK-8) and the increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to CCK-8 was determined. The data presented suggest that the three residues are indeed involved in antagonist binding. However, the relatively small changes in receptor affinity and activity disfavour a role for hydrogen bonds as a driving force.

2. Materials and methods

2.1. Mutagenesis of CCK_A receptor cDNA

Full-length cDNA encoding the rat CCK_A receptor, truncated to within three nucleotides of the first in frame ATG (Yule et al., 1993) and subcloned into the mammalian expression vector pTEJ8, was kindly provided by Dr. C.D. Logsdon (University of Michigan, Ann Arbor, MI, USA). Oligonucleotides for mutagenesis are 5′-CTACTTCATGGGTACCGCCGTGAGCGTTT-3′ (S139A), 5′-CCTACACCTCCGCCTGTGTGAACCC-CATCATC-3′ (S379A) and 5′-ATCTTCAGCGCTG-CAGCCTGGCGG-3′ (N349A). Mutations were introduced by the T7-GEN site-directed mutagenesis kit from US Biochemical (Cleveland, OH, USA) and confirmed by sequencing. For transfection, cDNA was subcloned into the *Hin*dIII and *Bam*HI sites of the mammalian expression vector pTEJ8.

2.2. Transfection of CHO cells with cDNA of the CCK_A receptor

Chinese hamster ovary (CHO)-K1 cells were transfected with cDNA of the CCK_A receptor essentially as described in 'Methods in Electroporation' (Bio-Rad). With this method, transfection efficiencies of 50% to 100% have been reported in CHO cells as judged from β-gal expression and/or immunocytochemistry. Briefly, CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C. For transfection, CHO cells were grown to 70% confluency, trypsinized and transferred to a cuvette (3×10^6 cells/300 μl). The cells were electroporated (250 V, 960 μF) in the presence of 20 μg of pTEJ8 containing full-length wild-type or mutated CCK_A receptor cDNA. Based on the ability of transfected cells to respond to a maximal concentration of CCK-8 with an increase in cytosolic free calcium concentration we routinely obtained a transfection efficiency of about 70%. Similar values were reached with the CCK_B receptor and the histamine receptor. In initial studies using immunocytochemistry following transfection with a pSG5-derived vector expressing the extracellular part of CD45 containing a vsv-tag a considerably lower value of 30% was obtained. This suggests that receptor proteins can already be detected at relatively low expression levels by virtue of their ability to efficiently stimulate second messenger production, whereas considerably higher expression levels are needed for immunocytochemical detection.

2.3. Fluorescence measurements in individual CHO cells

Transfected cells were grown for 24 h, trypsinized and seeded on a glass coverslip (2×10^4 cells/30 μl). The cells were allowed to attach for 30 min. Culture medium was added and the cells were grown to subconfluency for 24 h. For fluorescence measurements, the cells were incubated with 2 μM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxy methylester (fura-2/AM) for 30 min at 37°C. To remove non-hydrolysed dye, cells were washed 3 times with a physiological salt solution containing 137 mM NaCl, 4.7 mM KCl, 0.56 mM $MgCl_2$, 1.28 mM $CaCl_2$, 1.0 mM Na_2HPO_4, 2 mM L-glutamine, 5.5 mM D-glucose, 0.1% (w/v) bovine serum albumin and 10 mM HEPES (pH 7.4). Coverslips were mounted in a thermostatic (34°C) perfusion chamber, placed on the stage of an inverted microscope (Nikon Diaphot). Superfusion was at a flow rate of 1 ml/min. Routinely, an epifluorescent 40× magnification oil immersion objective was used to allow simultaneous monitoring of an average of close to 80 individual cells. Dynamic video imaging was carried out as described previously (Willems et al., 1993a) using the MagiCal hardware and TARDIS software provided by Joyce Loebl (Dukesway, Team Valley, Gateshead, UK). The fluorescence emission ratio at 492 nm was monitored as a measure of $[Ca^{2+}]_i$ after excitation at 340 and 380 nm.

2.4. Fluorescence measurements in suspensions of CHO cells

Transfected CHO cells were seeded in 25 cm^2 culture flasks (1×10^6 cells/flask) and grown for 48 h. The cells

were trypsinized and washed twice in a HEPES/Tris medium containing 133 mM NaCl, 4.2 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.8 mM glucose, 0.2 mg/ml soybean trypsin inhibitor, an amino acid mixture according to Eagle, 0.1% (w/v) bovine serum albumin and 10 mM HEPES, adjusted with Tris to pH 7.4. Cells were resuspended in HEPES/Tris medium containing 1% (w/v) bovine serum albumin and loaded with 2 μM fura-2/AM for 20 min at 37°C. Excess fura-2/AM was removed by washing the cells twice with HEPES/Tris medium containing 0.1% (w/v) bovine serum albumin. Cells were transferred to a cuvette placed in a Shimadzu RF-5000 spectrofluorophotometer equipped with a magnetic stirrer and a thermostated cuvette holder. Fluorescence measurements were carried out at 37°C as described previously (Willems et al., 1993a,b). The fluorescence emission ratio at 490 nm was monitored as a measure of the average $[Ca^{2+}]_i$ after excitation at 340 and 380 nm.

2.5. Radioligand binding studies

Transfected CHO cells, cultured for 48 h, were washed twice with 20 mM sodium phosphate buffer (pH 7.8). After scraping, the cells were resuspended in 50 mM Tris/HCl (pH 7.8) containing 0.3 mg/ml soybean trypsin inhibitor and freeze-thawed 3 times using liquid nitrogen. The suspension was centrifuged at $10\,000 \times g$ (Eppendorf minifuge) for 1 h at 4°C. The pellet was resuspended in 50 mM Tris/HCl (pH 7.8). Binding studies were performed as described previously (IJzerman and Melman, 1992). Briefly, 100 μl of membrane suspension (40 μg protein) were added to 300 μl of an assay buffer containing (final concentrations) 3.75 mM $MgCl_2$, 0.0375% (w/v) 3-[(3-cholamidopropyl) dimethylammonio] 1-propanesulfonate, 50 pM [^{125}I]CCK-8, the indicated concentration of devazepide (L-364.718) and 7.5 mM HEPES (pH 7.4) and incubated for 30 min at 37°C. The reaction was quenched by the addition of 1 ml of ice-cold 'stop solution' containing 5 mM $MgCl_2$ and 10 mM HEPES (pH 7.4) followed by rapid filtration through pre-soaked Whatman GF/B filters. The filters were washed 3 times with 2 ml ice-cold stop solution and taken up in scintillation fluid. Radioactivity retained on the filters was determined by liquid scintillation counting. Total binding and non-specific binding were determined with 0.1 pM and 0.1 μM CCK-8, respectively. Values presented are expressed as percentage of specific binding ([total binding] − [non-specific binding]).

2.6. Data analysis and statistics

The results presented are the mean ± S.E. of the number of experiments indicated in the text. Paired Student's *t*-tests were used to determine statistical differences ($P < 0.05$). Half-maximal CCK-8 concentrations for the recruitment of individual cells in terms of Ca^{2+} mobilization and the increase in $[Ca^{2+}]_i$ in suspensions of cells and half-maximal devazepide concentrations for the inhibition of [^{125}I]CCK-8 binding were calculated by means of the nonlinear regression computer program InPlot (Graphpad Software for Science, San Diego, CA, USA).

2.7. Materials

Tissue culture medium and additives were purchased from Gibco BRL (Paisley, UK) and fura-2/AM from Molecular Probes (Eugene, OR, USA). Soybean trypsin inhibitor and CCK-8 were obtained from Sigma Diagnostics (St. Louis, MO, USA) and devazepide (L-364,718) from Merck (Darmstadt, Germany). [^{125}I]CCK-8 (2200 Ci/mmol) was purchased from NEN Research Products (Boston, MA, USA) and GF/B filters from Whatman (Maidstone, UK). All other chemicals were of reagent grade.

3. Results

3.1. Effect of receptor mutations on the inhibition by devazepide of the specific binding of [^{125}I]CCK-8 to membranes of CHO cells transiently expressing the CCK_A receptor

Devazepide inhibited specific binding of radiolabelled CCK-8 to wild-type membranes dose-dependently. The IC_{50} was calculated to be 0.96 nM (S.E. = 0.11; $n = 5$) (Fig. 1). Similarly, devazepide inhibited specific binding of [^{125}I]CCK-8 to membranes of the S139A and S379A mutant with IC_{50} values of 1.33 nM (S.E. = 0.25; $n = 5$) and 0.73 nM (S.E. = 0.04; $n = 5$), respectively. Although the

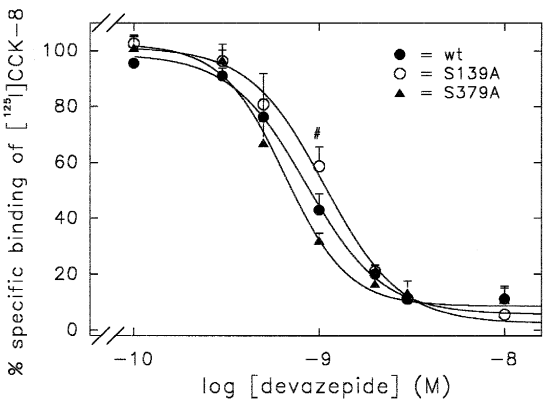

Fig. 1. Inhibition of [^{125}I]CCK-8 binding to membranes from CHO cells transiently expressing wild-type or mutant CCK_A receptors by devazepide. Membranes from CHO cells transiently expressing wild-type or mutant CCK_A receptors were incubated with 50 pM [^{125}I]CCK-8 in the presence of various concentrations of devazepide for 30 min at 37°C. For each membrane preparation, specific binding, i.e. the difference between maximal binding in the presence of 0.1 pM CCK-8 and aspecific binding in the presence of 0.1 μM CCK-8, is set at 100%, to which all other values, after correction for aspecific binding, are related. # Significantly different from the mutant S379A receptor.

difference between the latter two IC_{50} values is small, it is statistically significant. On the other hand, both values are not significantly different from that obtained with the wild-type receptor. Compared to wild-type membranes, specific labelling, expressed in cpm per mg of protein, with membranes from the mutant receptors S139A, S379A and N349A and mock-transfected cells amounted to 118.0% (S.E. = 24.4; $n = 4$), 75.3% (S.E. = 5.0; $n = 4$), 2.6% ($n = 1$) and 0.3% (S.E. = 1.0; $n = 3$), respectively. The low value obtained with the N349A mutant receptor suggests that affinity and/or expression of this receptor is markedly reduced. No specific binding was observed with mock-transfected CHO-K1 cells.

3.2. Effect of receptor mutations on the inhibition by devazepide of the recruitment of CHO cells transiently expressing the CCK_A receptor in terms of CCK-8-evoked Ca^{2+} mobilization

CCK-8 evoked a rapid increase in $[Ca^{2+}]_i$ in CHO-K1 cells transiently expressing the wild-type CCK_A receptor (Fig. 2). CCK-8 increased the number of responding cells dose-dependently ($EC_{50} = 78$ pM) to a maximum of 68% (S.E. = 7.5, $n = 5$) reached with 10 nM CCK-8 (Fig. 3, upper left). The number of responding cells remained unchanged upon a further increase in the CCK-8 concentration (data not shown). In each experiment, the number of cells responding to 10 nM CCK-8 was set at 100%, to which all other values were correlated. These cells are referred to as CCK-8-recruitable cells. Similarly to the wild-type receptor, cells transfected with the mutant S139A receptor (Fig. 3, upper right) or the mutant S379A receptor (Fig. 3, lower left) were recruited by CCK-8 in a dose-dependent manner (EC_{50} values of 61 pM and 101 pM, respectively). Both EC_{50} values did not significantly differ from that obtained with the wild-type receptor, neither did the number of CCK-8-recruitable cells. In contrast, the

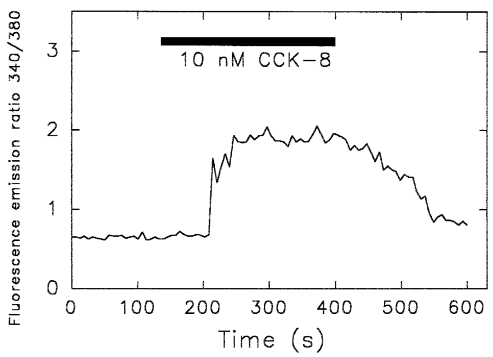

Fig. 2. CCK-8-induced increase in free cytosolic Ca^{2+} concentration in a single CHO cell transiently expressing the wild-type CCK_A receptor. CHO cells, transiently expressing wild-type CCK_A receptors, were loaded with fura-2 and changes in fluorescence emission ratio 340/380 nm, reflecting changes in $[Ca^{2+}]_i$, were monitored by digital imaging microscopy. The cells were superfused with physiological salt solution and stimulated with 10 nM CCK-8 for the indicated period of time.

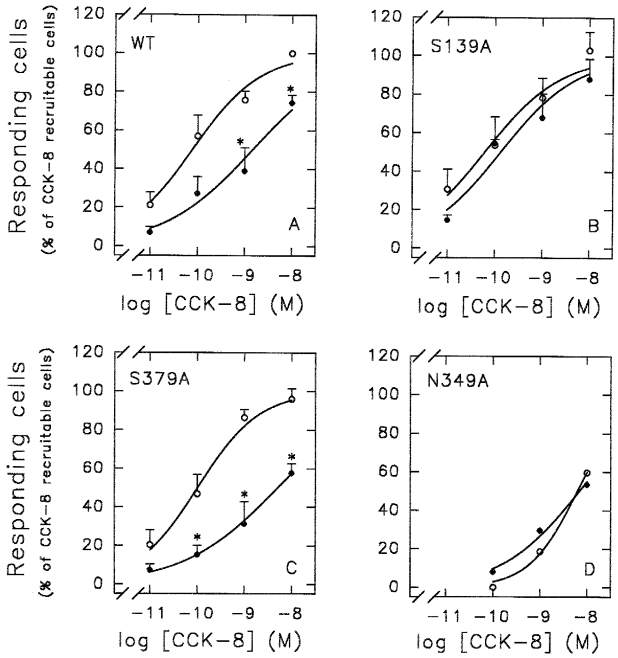

Fig. 3. Effect of devazepide on the dose-response curve for the CCK-8-induced recruitment of individual CHO cells transiently expressing wild-type or mutant CCK_A receptors. CHO cells, transiently expressing wild-type or mutant CCK_A receptors, were loaded with fura-2 and superfused with physiological salt solution containing either 3 nM devazepide (closed circles) or 0.1% (v/v) dimethyl sulfoxide (open circles). At 1 min, the cells were stimulated with the indicated concentrations of CCK-8 for 5 min. In each experiment, the number of wild-type-transfected cells displaying an increase in free cytosolic Ca^{2+} concentration in response to stimulation with 10 nM CCK-8, referred to as CCK-8 recruitable cells, is set at 100%, to which all other values are related. Upper panels: (left) wild-type receptor, (right) mutant S139A receptor; lower panels: (left) mutant S379A receptor and (right) mutant N349A receptor. The values presented are the mean ± S.E. of four transfections. In case of the mutant N349A receptor the values presented are the mean of two transfections. * Significantly different from corresponding control ($P < 0.05$).

EC_{50} value obtained with the mutant N349A receptor was 80-fold increased to 6.2 nM (Fig. 3, lower right). The latter value was calculated under the assumption that with higher CCK-8 concentrations the 100% value would be reached.

Devazepide (3 nM), when added 1 min prior to stimulation with CCK-8, caused the dose-recruitment curve of cells transfected with the wild type CCK_A receptor to shift to the right (Fig. 3, upper left). The EC_{50} value was calculated to be increased 19-fold to a value of 1.5 nM. With the mutant S379A receptor an even higher (50-fold) increase in the EC_{50} value was observed to a value of 5.1 nM (Fig. 3, lower left). By contrast, cells transfected with the mutant S139A receptor were relatively insensitive to 3 nM devazepide (Fig. 3, upper right). The EC_{50} value was calculated to be 0.13 nM, representing a 2-fold increase only. The EC_{50} value obtained with the mutant N349A receptor remained virtually unchanged in the presence of the receptor antagonist (EC_{50} values of 6.2 nM and 6.9 nM CCK-8 in the absence and presence of 3 nM devazepide, respectively) (Fig. 3, lower right). Again, both values were

calculated under the assumption that with higher CCK-8 concentrations the 100% value would be reached. Unfortunately, such higher CCK-8 concentrations could not be tested since they evoked a rise in $[Ca^{2+}]_i$ in part of the non-transfected CHO cells. The latter response was potently inhibited by devazepide suggesting the involvement of a CCK_A type of receptor. Fig. 4, in which for each single CCK-8 concentration the number of cells responding in the presence of devazepide is expressed as a percentage of that responding in the absence of the antagonist, shows that with the S139A mutant devazepide did not affect the number of cells recruited by 0.1 nM CCK-8, whereas with the wild-type receptor and the S379A mutant devazepide inhibited recruitment by 40% and 70%, respectively. At 0.1 nM and 1 nM CCK-8, the percentage recruitable cells responding in the presence of devazepide was significantly less with the mutant S379A receptor as compared to the mutant S139A receptor.

3.3. Effect of receptor mutations on the inhibition by devazepide of the CCK-8-evoked peak increase in average $[Ca^{2+}]_i$ in a suspension of CHO cells transiently expressing the CCK_A receptor

CCK-8 transiently increased the fluorescence emission ratio in a suspension of fura-2-loaded CHO cells transfected with the wild-type CCK_A receptor, reflecting a transient increase in average $[Ca^{2+}]_i$ ($[Ca^{2+}]_{i,av}$). The peak value of the transient increased with increasing of the CCK-8 concentration to reach a maximum at 10 nM CCK-8 (data not shown). In each experiment, the latter value was set at 100%, to which all other values were correlated. With the wild-type receptor the EC_{50} value was calculated to be 113 pM (Table 1). The peak increase in $[Ca^{2+}]_{i,av}$ evoked by 10 nM CCK-8 was not significantly

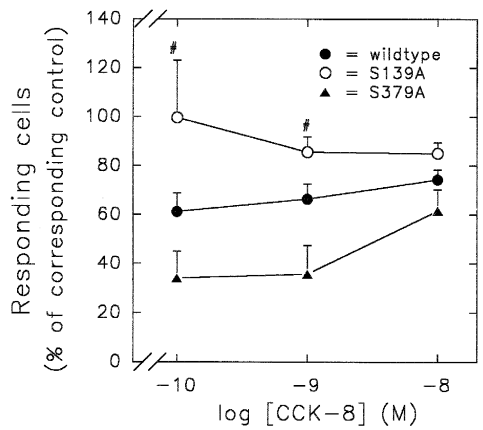

Fig. 4. Relative inhibition of CCK-8-induced cell recruitment at differing CCK-8 concentrations by devazepide. For each individual CCK-8 concentration the number of cells responding in the presence of devazepide is expressed as percentage of the number of cells responding in the absence of the antagonist. The data presented are obtained from the experiments described in the caption of Fig. 3. # Significantly different from the mutant S379A receptor ($P < 0.05$).

Table 1
Summary of the EC_{50} values for the CCK-8-evoked increase in cytosolic free Ca^{2+} concentration obtained with wild-type and mutant CCK_A receptors in cell recruitment studies and cell suspension measurements performed in the absence and presence of devazepide

Receptor	− Devazepide	+ Devazepide	Ratio
Cell recruitment studies			
WT	78 pM	1.5 nM	19×
S139A	61 pM	0.13 nM	2×
S379A	101 pM	5.1 nM	50×
N349A	6.2 nM	6.9 nM	1×
Cell suspension measurements			
WT	113 pM	25.8 nM	228×
S139A	80 pM	4.6 nM	58×
S379A	405 pM	0.27 μM	667×
N349A	8.2 nM	0.66 μM	80×

different between cells expressing the wild-type receptor and cells expressing the mutant S139A receptor or the mutant S379A receptor but was reduced by 50% in cells expressing the mutant N349A receptor. With the S139A mutant the EC_{50} value was calculated to be decreased 0.7-fold to 80 pM, whereas with the S379A mutant this value was calculated to be increased 3.6-fold to 405 pM. With the N349A mutant the EC_{50} value was calculated to be increased 73-fold to 8.2 nM, under the assumption, however, that with higher CCK-8 concentrations the 100% value will be reached.

Devazepide (3 nM), when added 3 min prior to stimulation with CCK-8, caused the dose-response curve of cells transfected with the wild-type CCK_A receptor to shift to the right. The peak increase obtained with 10 nM CCK-8 was reduced by 60%. Under the assumption that with higher CCK-8 concentrations the 100% value will be reached, the EC_{50} value was calculated to be increased 228-fold to 25.8 nM (Table 1). With the mutant S379A receptor the peak increase obtained with 10 nM CCK-8 was reduced by 75% and the EC_{50} value was calculated to be increased 667-fold to 0.27 μM. With the mutant S139A receptor the peak increase obtained with 10 nM CCK-8 was reduced by 40% and the EC_{50} value was calculated to be 4.6 nM, representing a 58-fold increase only. Devazepide effectively inhibited the CCK-8-induced peak increase in $[Ca^{2+}]_{i,av}$ in cells expressing the mutant N349A receptor. In the latter cells the peak increase obtained with 10 nM CCK-8 was reduced by 85% and the EC_{50} value was calculated to be increased 80-fold to 0.66 μM.

4. Discussion

The aim of the present study was to investigate by site-directed mutagenesis the possible involvement of the hydrophilic residues Ser^{139}, Asn^{349} and Ser^{379} in the binding of the CCK_A receptor antagonist devazepide. Func-

tional characteristics of the three mutants were compared to the wild-type receptor in three experimental setups, (i) inhibition of radioligand binding, (ii) inhibition of the CCK-8-evoked increase in $[Ca^{2+}]_{i,av}$ in a suspension of cells, and (iii) inhibition of CCK-8-evoked recruitment of individual cells in terms of receptor-mediated Ca^{2+} mobilization.

In radioligand binding studies replacing Ser^{139} by alanine slightly reduced devazepide binding. In contrast, the S379A mutation appeared to increase the affinity of devazepide for the CCK_A receptor. Although the IC_{50} values obtained with both mutants did not statistically differ from the value obtained with the wild-type receptor, they did so from one another. With the N349A mutant both agonist and antagonist binding were drastically decreased, suggesting that this amino acid may be important in proper receptor expression.

In addition to studying the effect of the receptor mutations on the ability of devazepide to displace radiolabelled CCK-8 in a membrane preparation, we investigated the effect of these mutations on the ability of devazepide to inhibit the process of receptor-mediated Ca^{2+} mobilization in intact cells. Basically, the same results were obtained with both approaches. Thus, compared to the wild-type receptor devazepide inhibited CCK-8-evoked Ca^{2+} mobilization less effective with the mutant S139A and N349A receptor and more effective with the mutant S379A receptor. From the observation that the receptor mutations affected the ability of devazepide to displace radiolabelled CCK-8 in a membrane preparation and inhibit the process of receptor-mediated Ca^{2+} mobilization in intact cells in the same way it can be concluded that these mutations did not interfere with the process of signal transduction.

Beside suspension measurements we performed cell recruitment studies to analyse the effect of receptor mutations on devazepide inhibition of receptor-mediated Ca^{2+} mobilization. The advantage of this approach, which is based on the observation that agonists dose-dependently increase the number of responding cells (Willems et al., 1993a, 1995; Smeets et al., 1996), is that only cells that express the receptor are included. The present study shows that with CHO cells transiently expressing the CCK_A receptor a transfection efficiency of close to 70% can be reached. Another benefit of this approach is that cells grown on coverslips do not have to be isolated and therefore are not at risk of being exposed to conditions that might damage the receptor. The present study clearly demonstrates that this approach can be of use to analyse the effect of receptor mutations on the relative ability of antagonists to inhibit agonist-evoked Ca^{2+} mobilization.

The data presented are in agreement with the idea that the three amino acids investigated in this study form part of the putative antagonist binding site, though most probably none of them in the role of hydrogen acceptor.

This is the first report on the use of mutant receptors to address the antagonist binding site of the CCK_A receptor. However, the importance of two other amino acids forming part of the proposed antagonist binding site in devazepide binding was recently demonstrated in a study in which CCK_B receptor amino acids were replaced by the corresponding amino acids from the CCK_A receptor (Kopin et al., 1995; Jagerschmidt et al., 1996). For instance, when His^{381} in the rat CCK_B receptor and its equivalent on position 376 in the human CCK_B receptor were mutated to leucine, the corresponding amino acid on position 372 in the CCK_A receptor, the affinity for devazepide was increased (Kopin et al., 1995; Jagerschmidt et al., 1996). Similarly, when Val^{349} in the human CCK_B receptor and its equivalent on position 353 (position 354 according to Wank et al., 1992) in the rat CCK_B receptor were mutated to isoleucine, the corresponding amino acid on position 345 in the CCK_A receptor, devazepide binding was increased (Kopin et al., 1995; Beinborn et al., 1993; Malatynska et al., 1995). This suggests that these two amino acids, Ile^{345} and Leu^{372}, form indeed part of the proposed devazepide binding site. In the same way, the involvement of the amino acids Thr^{133}, Gln^{225} and Phe^{346}, located close to the proposed antagonist binding site in the rat CCK_A receptor, in devazepide binding was demonstrated (Kopin et al., 1995). Thus far, only one CCK_B receptor mutation has been described (Arg^{57} changed to its corresponding amino acid Gln^{59} in the rat CCK_A receptor) outside the proposed CCK_A receptor antagonist binding site that affected devazepide binding (Kopin et al., 1995).

Taken together the data reported in the literature and those obtained in the present study are in agreement with the proposed antagonist binding site of the CCK_A receptor.

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