Exploressing the Binding Site Crevice of a Family B G Protein-Coupled Receptor, the Type 1 Corticotropin Releasing Factor Receptor

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ABSTRACT

Family B of G protein-coupled receptors (GPCRs) is composed of receptors that bind peptides, such as secretin, glucagon, parathyroid hormone, and corticotropin releasing factor (CRF), which play critical physiological roles. These receptors, like all GPCRs, share a common structural motif of seven membrane-spanning segments, which have been proposed to bind small ligands, such as antalarmin, a nonpeptide antagonist of the type 1 receptor for CRF (CRF1). This leads to the hypothesis that as for family A GPCRs, the binding sites of small ligands for family B GPCRs are on the surface of a water-accessible crevice, the binding-site crevice, which is formed by the membrane-spanning segments and extends from the extracellular surface of the receptor into the plane of the membrane. To test this hypothesis we have begun to obtain structural information about family B GPCRs, using as a prototype the CRF1, by determining the ability of sulfhydryl-specific methanethiosulfonate derivatives, such as the methanethiosulfonate-ethylammonium (MTSEA), to react with CRF1 and thus irreversibly inhibit 125I-Tyr0-sauvagine binding. We found that MTSEA inhibited 125I-Tyr0-sauvagine binding to CRF1 and that antalarmin protected against this irreversible inhibition. To identify the susceptible cysteine(s), we mutated, one at a time, four endogenous cysteines to serine. Mutation to serine of Cys211, Cys233, or Cys364 decreased the susceptibility of sauvagine binding to irreversible inhibition by MTSEA. Thus, Cys211, Cys233, and Cys364 at the cytoplasmic ends of the third, fourth, and seventh membrane-spanning segments, respectively, are exposed in the binding site crevice of CRF1.

Introduction

Family B of G-protein-coupled receptors (GPCRs) is composed of receptors that bind functionally important peptides, including corticotropin releasing factor (CRF), a 41-amino acid peptide that plays a major physiological role by regulating the activity of the hypothalamic-pituitary-adrenal axis (Vale et al., 1981; Chrousos, 1995; Harmar, 2001). Sequence analysis of these receptors has revealed seven putative, mostly hydrophobic, plasma membrane-spanning segments connected by alternating intracellular and extracellular loops (Gether, 2000; Grigoriadis et al., 2001; Harmar, 2001). These receptors also have a large extracellular amino-terminal region (N-region) that has been structurally characterized in NMR and crystallographic studies (Grace et al., 2007; Pioszak et al., 2008; Underwood et al., 2010). The N-region and the extracellular loops of family B GPCRs have been shown to play an important role in peptide binding (Holtmann et al., 1996; Liaw et al., 1997; Perrin et al., 1998; Dautzenberg et al., 1999; Unson et al., 2002; Kraetke et al., 2005a; Grace et al., 2007; Assil-Kishawi et al., 2008; Pioszak et al., 2008; Gkountelias et al., 2009).

In contrast to the extracellular regions, little is known about the potential role in ligand binding of the membrane-spanning segments (TMs) of family B GPCRs. Although the TMs have been proposed to bind small nonpeptide ligands, such as antalarmin, an antagonist for the type 1 CRF receptor (CRF1), the specific interactions have not been identified (Liaw et al., 1997; Hoare et al., 2003). In contrast to the TMs of family A GPCRs, which have been structurally characterized in multiple crystallographic, biophysical, and biochemical studies, little is known about the potential role in ligand binding of the membrane-spanning segments of family B GPCRs. This research project was supported by a National Alliance for Research on Schizophrenia and Depression (NARSAD) Young Investigator Award (to G.L.).

Abbreviations: GPCR, G protein-coupled receptor; CRF, corticotropin-releasing factor; CRF1, type 1 receptor for the corticotropin releasing factor; TM, transmembrane; MTS, methanethiosulfonate; MTSEA, methanethiosulfonate-ethylammonium; MTSET, methanethiosulfonate-trimethylammonium; MTSES, methanethiosulfonate-ethylsulfonate; WT, wild type; HEK, human embryonic kidney; PBS, phosphate-buffered saline; ANOVA, analysis of variance.
rational studies, there is no structural information about the TMs of family B GPCRs, further complicating a determination of their role in ligand binding. It is noteworthy that family B GPCRs display very little sequence similarity with family A receptors, and their TMs do not share the common structural/functional motifs identified in the latter (Donnelly, 1997; Frimurer and Bywater, 1999; Gether, 2000). All of these factors hinder the construction of accurate molecular models of family B GPCRs. Nevertheless, based on sequence analysis, several models of these receptors have been created (Donnelly, 1997; Frimurer and Bywater, 1999). Despite the assumptions required and the associated uncertainties of the exact boundaries of the TMs and their orientations, the overall TM packing of family B GPCRs has been proposed to be similar to that of family A GPCRs. This, in conjunction with their role in the binding of small nonpeptide ligands, led us to hypothesize that as in the family A GPCRs, the TMs of family B receptors form a water-filled binding-site crevice, which extends from the extracellular surface of the receptor into the plane of the membrane (Javitch et al., 1994). The surface of this crevice is formed by residues that contact ligands and by other residues that may play a structural role and affect binding indirectly.

To test this hypothesis, we sought to obtain structural information for the TMs of family B GPCRs, using as a prototype the CRF₁. Our starting point was to test whether one or more of the endogenous TM cysteines in CRF₁ face the putative binding site crevice by determining their accessibility for reaction with small, charged, sulfhydryl-specific methanethiosulfonate (MTS) derivatives. These reagents react vastly faster with water-accessible sulfhydryl groups of many receptors, including CRF₁, than with sulfhydryls buried in the protein or facing the lipid bilayer (Karlin and Akabas, 1998). Using this method, previous studies have revealed the presence of endogenous TM cysteines that face the binding site crevice of various family A GPCRs (Javitch et al., 1994; Deng et al., 2000). Here, we found that the endogenous Cys211, Cys233, and Cys364 in the third, fourth, and seventh membrane-spanning segments of CRF₁ are located on the surface of a binding-site crevice of CRF₁, being accessible to the reaction with charged polar MTS reagents and protected from this reaction by bound antalarmin.

**Materials and Methods**

**Plasmids and Site-Directed Mutagenesis.** The cDNA sequence encoding CRF₁ was subcloned into the bicistronic expression vector pCIn4, thereby creating the vector pCIn4-CRF₁ (Gkountelias et al., 2009). Serine mutations were generated by the polymerase chain reaction-mediated mutagenesis, using Pfu polymerase (MBI Fermentas, Hanover, MD) and mutagenic oligonucleotides encoding the desired amino acid substitution. The polymerase chain reaction-generated DNA fragments containing the mutations were subcloned into the pCIn4-CRF₁ plasmid, and the mutations were confirmed by DNA sequencing. Mutants are named as (wild-type residue)X(residue number)X(seine), where the residues are given in the single-letter code.

**Cell Culture, Transfection, and Harvesting.** Human embryonic kidney (HEK) 293 cells were grown in Dulbecco’s modified Eagle’s medium/F-12 (1:1) containing 3.5 g/l glucose and 10% bovine calf serum at 37°C and 5% CO₂. Sixty-millimeter dishes of HEK 293 cells at 80 to 90% confluence were transfected with 2 to 3 mg of wild-type (WT) or mutant pCIn4-CRF₁ using 9 µl of Lipofectamine and 2 ml of Opti-MEM (both from Invitrogen, Carlsbad, CA). To generate stably transfected pools of cells expressing the receptors 5 to 12 h after transfection, the medium was replaced by Dulbecco’s modified Eagle’s medium/F-12 (1:1) containing 3.5 g/l glucose, 10% bovine calf serum (HyClone Laboratories, Logan, UT) and 700 µg/ml G418 (Geneticin), and antibiotic (Invitrogen). The antibiotic was added to select a stably transfected pool of cells. Cells stably expressing WT or CRF₁ mutants, at 100% confluence in 60- or 100-mm dishes, were washed with phosphate-buffered saline (PBS) (4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl, pH 7.3–7.4, at 37°C), briefly treated with PBS containing 2 mM EDTA (PBS/EDTA), and then dissociated in PBS/EDTA. Cell suspensions were centrifuged at 50 g for 2 min at room temperature, and the pellets were resuspended in 1 ml of buffer M (25 mM HEPES containing 5.4 mM KCl, 140 mM NaCl, and 2 mM EDTA, pH 7.2, at 22–25°C) for treatment with MTS reagents or in 1.5 ml of buffer H (20 mM HEPES, containing 10 mM MgCl₂, 2 mM EGTA, 0.2 mg/ml bacitracin, and 0.93 µg/ml aprotinin, pH 7.2, at 4°C) for binding assays.

**125I-Tyr₀-Sauvagine Binding.** For radioligand binding assays, cell suspensions (1.5 ml) in buffer H were homogenized using an Ultra Turrax T25 homogenizer (IKA Janke and Kunkel, Staufen, Germany) at setting −20 for 10 to 15 s, at 4°C. The homogenates were centrifuged at 16,000g for 10 min at 4°C, and the membrane pellets were resuspended in 1 ml of buffer B (buffer H containing 0.1% bovine serum albumin, pH 7.2, at 20°C). The membrane suspensions were diluted in buffer B and used for homologous competition binding studies as described previously (Gkountelias et al., 2009). In brief, aliquots of diluted membrane suspensions (50 µl) were added into low retention tubes (Kisker-Biotech, Steinfurt, Germany), containing buffer B and 20 to 25 pM 125I-Tyr₀-sauvagine with or without increasing concentrations of Tyr₀-sauvagine (American Peptide Co., Sunnyvale, CA). The mixtures were incubated at 20 to 21°C for 120 min and then filtered using a Brandel cell harvester through Whatman 934AH glass fiber filters presoaked for 1 h in 0.3% polyethyleneimine at 4°C. The filters were washed three times with 0.5 ml of ice-cold PBS, pH 7.1, containing 0.01% Triton X-100. Filters were assessed for radioactivity in a gamma counter (1275 mini-gamma, 80% efficiency; LKB Wallac, Chalfont St. Giles, Buckinghamshire, UK). The amount of membrane used was adjusted to ensure that the specific binding was always equal to or less than 10% of the total concentration of the added radioligand. Specific 125I-Tyr₀-sauvagine binding was defined as total binding less nonspecific binding in the presence of 500 to 1000 nM CRF. The Kᵦ values for 125I-Tyr₀-sauvagine binding were determined by analyzing homologous competition data with Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

**Reactions with MTS Reagents.** For treatment with MTS reagents, aliquots (0.1 ml) of cell suspensions in buffer M were incubated with the MTS reagents MTS-ethylaminesulfonate (MTSEA), MTS-ethyltrimethylenemmonium (MTEX), or MTS-ethylsulfonate (MTSES) at the stated concentrations for 15 s to 22 to 25°C. Cell suspensions were then diluted 140-fold in buffer PBS/EDTA, pH 7.1, at 22 to 25°C containing 10 mM MgCl₂, centrifuged at 250g for 5 min at 22 to 25°C, and the pellets were resuspended in 1 ml of buffer M containing 10 mM MgCl₂. Cell suspensions were centrifuged at 250g for 5 min at 22 to 25°C, and the pellets were homogenized in 1.5 ml of buffer H, as described above. The homogenates were centrifuged at 16,000g for 10 min at 4°C and the membrane pellets were resuspended in 1 ml of buffer B (buffer H containing 0.1% bovine serum albumin, pH 7.2, at 20°C). The membrane suspensions were used to assay for 125I-Tyr₀-sauvagine binding as described above.

Protection experiments were performed by preincubation of aliquots (0.1 ml) of cell suspensions with increasing concentrations of the nonpeptide CRF₁-selective antagonist (1-1000 nM) antalarmin for 40 min at 37°C in a final volume of 1 ml of buffer M. Thereafter, the mixtures were centrifuged at 250g for 5 min at 22 to 25°C, and 0.9 ml of supernatant was removed by aspiration. The cell pellets were resuspended in the remaining 0.1 ml of supernatants, and the...
mixtures were treated with 2.5 mM MTSEA as described above. Cells were subsequently diluted 140-fold in buffer PBS/EDTA, pH 7.1, at 22 to 25°C containing 10 mM MgCl₂, washed twice by centrifugation with buffer M containing 10 mM MgCl₂, and membrane homogenates were prepared and used to assay for 125I-Tyr⁰-sauvagine binding as described above.

Results

Reaction of MTS Reagents with the CRF₁. To assess for the reaction of MTS reagents with the CRF₁, we treated intact HEK 293 cells stably expressing WT CRF₁ with MTSEA, MTSET, or MTSES and subsequently determined the specific binding of 125I-Tyr⁰-sauvagine in membrane homogenates.

Treatment of HEK 293 cells stably expressing CRF₁ with the positively charged MTSEA at a concentration of 2.5 mM significantly decreased the specific binding of 125I-Tyr⁰-sauvagine to CRF₁ (Fig. 1). In contrast to MTSEA, the bulkier MTSET (positively charged) and MTSES (negatively charged), at concentrations of 2.5 mM, did not significantly reduce the specific binding of 125I-Tyr⁰-sauvagine to CRF₁ (Fig. 1). As shown in Fig. 2, MTSEA treatment decreased the specific 125I-Tyr⁰-sauvagine binding in a dose-dependent manner with an IC₅₀ of 2.1 mM (−logIC₅₀ = 2.69 ± 0.18), reaching a plateau of approximately 20% residual specific binding. Longer treatment of CRF₁ with 10 mM MTSEA for 2 min demonstrated a similar plateau in radioligand binding (data not shown).

Mechanism of the Inhibitory Effect of MTSEA on Binding. To examine the mechanism of the inhibitory effect of MTSEA on 125I-Tyr⁰-sauvagine binding, we determined the binding affinity of 125I-Tyr⁰-sauvagine before and after the reaction of MTSEA with CRF₁. As shown in Fig. 3, treatment of WT CRF₁ with either 2.5 or 15 mM MTSEA failed to significantly affect 125I-Tyr⁰-sauvagine binding affinity. Thus, inhibition of 125I-Tyr⁰-sauvagine binding after the reaction of CRF₁ with MTSEA resulted from a reduction in the apparent number of functional binding sites, consistent with a loss of binding to receptor that was fully derivatized. A possible explanation for the small fraction of residual binding with normal affinity despite treatment with saturating MTSEA could be that a small proportion of receptors was located intracellularly and thus protected from the reagent, whereas the binding experiments were performed with membrane homogenates in which 125I-Tyr⁰-sauvagine labeled all receptors.

Protection of CRF₁ Against MTSEA Reaction. To test whether the small nonpeptide CRF₁-selective antagonist an-
talamin protected the WT CRF₁ against MTSEA reaction, we determined its ability to slow the reaction. We treated CRF₁-expressing cells, incubated with or without antalarmin, with MTSEA (2.5 mM), and after washing, we determined ¹²⁵I-Tyr⁰-sauvagine binding in membrane homogenates from these cells. As shown in Fig. 4, antalarmin protected CRF₁ against MTSEA reaction in a concentration-dependent manner, with complete protection at a high concentration of the ligand.

**Mutations of the Endogenous Cysteines of CRF₁.** To identify the endogenous cysteine(s) of CRF₁ that reacted with MTSEA to inhibit binding, we mutated to serine, one at a time, the endogenous Cys128, Cys211, Cys233, and Cys364 (thus creating the C128S, C211S, C233S, and C364S mutants, respectively). According to the predicted topology of CRF₁, these cysteines are located in the first (TM1), third (TM3), fourth (TM4), and seventh (TM7) membrane-spanning segments of receptor (Fig. 5) (Grigoriadis et al., 2001). Before probing the reaction of MTSEA with the C128S, C211S, C233S, and C364S constructs, we tested the effect of the mutations on the functional properties of CRF₁, by determining the binding affinities (−logKᵢ) of ¹²⁵I-Tyr⁰-sauvagine for WT and the mutant receptors in homologous competition experiments performed under equilibrium conditions in membrane homogenates from HEK 293 cells stably expressing the receptors. Substitution of serine for Cys128, Cys211, Cys233, and Cys364 did not significantly affect the binding affinity of ¹²⁵I-Tyr⁰-sauvagine for CRF₁ (Fig. 6), suggesting that the mutations did not substantially alter the functional properties of the receptor.

**Reaction of MTSEA with CRF₁ Mutants.** To identify the reactive cysteine(s) of CRF₁, we determined whether the binding of ¹²⁵I-Tyr⁰-sauvagine to C128S, C211S, C233S, and C364S mutants was sensitive to MTSEA. As shown in Fig. 7, mutation of Cys128 to serine did not affect the sensitivity of radioligand binding to MTSEA; the residual ¹²⁵I-Tyr⁰-sauvagine binding to C128S (52.5 ± 3.3%) after MTSEA treatment (2.5 mM) was not significantly different from that to WT CRF₁ (57.4 ± 2.9%). In marked contrast, mutation to serine of Cys211, Cys233, or Cys364 significantly reduced sensitivity to MTSEA; the residual binding of ¹²⁵I-Tyr⁰-sauvagine to C211S (73.3 ± 4.5%), C233S (75.9 ± 9.9%), and C364S (83.0 ± 9.4%) after MTSEA treatment (2.5 mM) was not significantly different, but each was significantly different from that of WT (57.4 ± 2.9%) (Fig. 7).

Because radiolabeled sauvagine has been shown to bind to the extracellular portion of CRF₁ and thus is located at some distance from the endogenous cysteines being derivatized, the effects on binding must be indirect. To explore the mechanism of inhibition, we determined the affinity of ¹²⁵I-Tyr⁰-sauvagine binding to C128S, C211S, C233S and C364S (and to the other mutants tested in this study, as described below) before and after MTSEA reaction. As was the case for WT, MTSEA reaction did not significantly affect the binding affinity of ¹²⁵I-Tyr⁰-sauvagine for C128S, C211S, C233S or C364S (Fig. 6). Thus, as for WT, the decrease of ¹²⁵I-Tyr⁰-sauvagine binding to C128S after MTSEA reaction was due to a reduction of the apparent number of binding sites. In addition, the much smaller affect of MTSEA reaction with C211S, C233S, or C364S on ¹²⁵I-Tyr⁰-sauvagine binding was not due to an MTSEA-associated enhancement of the affinity of residual radioligand binding.

**Fig. 4.** Protection of ¹²⁵I-Tyr⁰-sauvagine-specific binding by preincubation with varying concentrations of antalarmin. HEK 293 cells stably expressing the wild-type CRF₁ were preincubated with increasing concentrations (1–1000 nM) of the antagonist, antalarmin for 30 min at 37°C and subsequently treated with 2.5 mM MTSEA as described under Materials and Methods. Thereafter, the cells were homogenized, and the ability of membrane homogenates to bind ¹²⁵I-Tyr⁰-sauvagine was assayed as described under Materials and Methods. The dotted line represents the specific binding after treatment with 2.5 mM MTSEA in the absence of antalarmin. The specific binding (percentage of control) was compared to that of ¹²⁵I-Tyr⁰-sauvagine binding to untreated receptors (control). Means ± S.E. are shown from six independent experiments, each performed with duplicate determinations.

**Fig. 5.** Snake plot representation of CRF₁ showing the approximate positions of its 13 cysteines. The cylinders represent the membrane-spanning segments (TM1–TM7) of CRF₁. Four endogenous cysteines (Cys128, Cys211, Cys233, and Cys364) are located in the membrane-spanning segments of CRF₁, whereas one is positioned in the first intracellular loop (Cys150) of receptor. Six endogenous cysteines are located in the extracellular N-region (Cys30 and Cys54, Cys44 and Cys87, and Cys68 and Cys102), forming three disulfide bridges (dotted lines) (Ploszak et al., 2008). The resting two endogenous cysteines (Cys188 and Cys258) are positioned in the first and second extracellular loops of CRF₁, and form a disulfide bond (dotted line) (Qi et al., 1997).
MTSEA Reaction with ΔCys Mutants. Based on the finding that substitution of Ser for a single Cys at positions 211, 233 or 364 rendered the receptor much less sensitive to MTSEA, we hypothesized that reaction with MTSEA of only a single one of these Cys, would not reduce sauvagine binding to CRF₁. To test this hypothesis, and even more importantly to create a suitable MTSEA-insensitive background construct for subsequent substituted-cysteine accessibility method studies, we mutated all the endogenous TM Cys (along with the cytoplasmic Cys150) to Ser, thereby creating the mutant ΔCys, which had a binding affinity for ¹²⁵I-Tyr⁰-sauvagine (−logKᵦ = 8.60 ± 0.63) similar to that of WT (−logKᵦ = 9.05 ± 0.08) (Fig. 8). As anticipated, simultaneous mutation of all the sensitive Cys in CRF₁ to Ser created a receptor less sensitive to 2.5 mM MTSEA; the residual binding of ¹²⁵I-Tyr⁰-sauvagine to ΔCys after MTSEA (2.5 mM) reaction was 90.9 ± 6.1% (Fig. 9). Consistent with our prediction, we found that addition of a single Cys into ΔCys (at positions, 128, 211, 233 or 364, thus creating the mutants ΔCys + 128C, ΔCys + 211C, ΔCys + 233C and ΔCys + 364C, respectively) failed to increase significantly the sensitivity to MTSEA. Thus, the residual binding of ¹²⁵I-Tyr⁰-sauvagine to ΔCys + 128C (83.1 ± 7.6%), ΔCys + 211C (86.0 ± 9.1%), ΔCys + 233C (89.4 ± 7.7%) and ΔCys + 364C (76.7 ± 7.3%) after MTSEA (2.5 mM) reaction was not significantly different from each other or from that of ΔCys (90.9 ± 6.1%), but it was significantly different from the corresponding binding to WT (57.4 ± 2.9%) (Fig. 9). These observations were not due to a mutation-associated or to an MTSEA-induced change of ¹²⁵I-Tyr⁰-sauvagine affinity, because the ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, and ΔCys + 364C mutants, similar to ΔCys, had normal ¹²⁵I-Tyr⁰-sauvagine affinity, which also was unaltered by MTSEA treatment (Fig. 8).

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**Fig. 6.** Binding affinity of Tyr⁰-sauvagine for C128S, C211S, C233S, and C364S receptors before and after MTSEA reaction. Suspensions of HEK 293 cells stably expressing WT CRF₁ or the mutants, C128S, C211S, C233S, or C364S were treated without (control) or with 2.5 mM MTSEA for 15 s at 22 to 25°C. Thereafter, the cells were homogenized and membrane homogenates were incubated with ¹²⁵I-Tyr⁰-sauvagine in the absence or presence of increasing concentrations of Tyr⁰-sauvagine. The data were fit to a one-site competition model by nonlinear regression and −logKᵦ values were determined as described under Materials and Methods. The bars represent the binding affinity (−logKᵦ) of ¹²⁵I-Tyr⁰-sauvagine before (□) or after MTSEA reaction (■). The mean ± S.E. values are from two to six independent experiments. The results were statistically analyzed using one-way ANOVA followed by least significant difference post hoc test. None of the mutations significantly altered ¹²⁵I-Tyr⁰-sauvagine affinity, and MTSEA treatment did not significantly alter radioligand affinity of any of the receptors tested.

**Fig. 7.** Effects of MTSEA reaction on specific ¹²⁵I-Tyr⁰-sauvagine binding to C128S, C211S, C233S, or C364S receptors. Suspensions of HEK 293 cells stably expressing WT CRF₁ or C128S, C211S, C233S, and C364S mutants were incubated for 15 s at 22 to 25°C with or without (control) 2.5 mM MTSEA. Thereafter, the cells were homogenized, and membrane homogenates were assayed for specific binding with ¹²⁵I-Tyr⁰-sauvagine, as described under Materials and Methods. The bars represent the specific binding (percentage of control), or residual binding, which is defined as the percentage of ¹²⁵I-Tyr⁰-sauvagine specific binding to MTSEA-treated receptor divided by the radioligand specific binding to the corresponding untreated receptor. The mean ± S.E. values are from 9 to 40 independent experiments, each performed with duplicate determinations. Asterisk indicates that the residual binding to C211S, C233S, or C364S mutant after MTSEA reaction was significantly different from the corresponding one to WT (P < 0.05, one-way analysis of variance and least significant difference post hoc test). In contrast, the residual binding to C211S, C233S, and C364S mutants after MTSEA reaction was not statistically different from each other.

**Fig. 8.** Binding affinity of Tyr⁰-sauvagine for ΔCys, ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, and ΔCys + 364C receptors before and after MTSEA reaction. Suspensions of HEK 293 cells stably expressing WT CRF₁ or the mutants, ΔCys, ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, or ΔCys + 364C were treated without (control) or with 2.5 mM MTSEA for 15 s at 22 to 25°C. Thereafter, the cells were homogenized and membrane homogenates were incubated with ¹²⁵I-Tyr⁰-sauvagine in the absence or presence of increasing concentrations of Tyr⁰-sauvagine. The data were fit to a one-site competition model by nonlinear regression, and −logKᵦ values were determined as described under Materials and Methods. The bars represent the binding affinity (−logKᵦ) of ¹²⁵I-Tyr⁰-sauvagine before (□) or after MTSEA reaction (■). The mean ± S.E. values are from two to six independent experiments. The results were statistically analyzed using one-way ANOVA followed by least significant difference post hoc test. None of the mutations considerably altered ¹²⁵I-Tyr⁰-sauvagine affinity and MTSEA treatment of all receptors tested did not significantly alter radioligand affinity.
We next hypothesized that derivatization of all three endogenous cysteines (Cys211, Cys233, and Cys364) is necessary for the reduction of sauvagine binding to CRF1 after MTSEA reaction. To test this hypothesis, we simultaneously added two or three cysteines (at positions 211, 233, and/or 364) into ΔCys and tested their ability to react with MTSEA. Simultaneous addition of two cysteines into ΔCys, in any combination, thus creating the mutants ΔCys + 233C + 364C, ΔCys + 211C + 364C, and ΔCys + 211C + 233C, did not significantly increase the sensitivity to MTSEA; although the residual binding of 125I-Tyr3-sauvagine to ΔCys + 233C + 364C (93.6 ± 13.0%), ΔCys + 211C + 364C (101.3 ± 9.9%), and ΔCys + 211C + 233C (96.8 ± 7.8%) after MTSEA (2.5 mM) reaction was not significantly different from each other or from ΔCys (90.9 ± 6.1%), it was significantly different from the corresponding binding to WT (57.4 ± 2.9%) (Fig. 10). In marked contrast, simultaneous addition of the three cysteines into the ΔCys, thus creating the mutant, ΔCys + 211C + 233C + 364C, synergistically increased the sensitivity of the mutant receptor to MTSEA, restoring the wild-type phenotype. The residual binding of 125I-Tyr3-sauvagine to ΔCys + 211C + 233C + 364C (61.6 ± 5.1%) after MTSEA treatment (2.5 mM) was not significantly different from the corresponding binding to WT (57.4 ± 2.9%), but it differed significantly from that to ΔCys (90.9 ± 6.1%) (Fig. 10). These findings were not due to a mutation-associated or to an MTSEA-induced change of 125I-Tyr3-sauvagine affinity, because the mutants had normal 125I-Tyr3-sauvagine affinity, which also was unaltered by MTSEA treatment (Fig. 11). These results also suggest that, similar to WT, the observed significant decrease of 125I-Tyr3-sauvagine binding to ΔCys + 211C + 233C + 364C after MTSEA reaction was due to a reduction of the apparent number of binding sites rather than to a decrease of its affinity.

**Discussion**

Reaction of MTSEA with CRF1, decreased the specific binding of the radiolabeled agonist 125I-Tyr3-sauvagine, suggesting that one or more endogenous cysteines was accessible for the reaction with the reagent. CRF1 contains 13 endogenous cysteines (Fig. 5). Four of these (Cys128, Cys211, Cys233, Cys364) are predicted to be in the TMs of CRF1 (TM Cys), one is intracellular (Cys150), whereas six (Cys30, Cys44, Cys54, Cys68, Cys87, and Cys102) are located in the extracellular N-region of CRF1, and form three disulfide bonds (Pioszak et al., 2008). In addition, two cysteines (Cys188, and Cys258) in the first and second extracellular loops, which are highly conserved among the G-protein coupled receptors, probably participate in the formation of a disulfide bond (Qi et al., 1997). Because the MTS reagents do not react with disulfide-bonded cysteines, and have limited access to intracellular cysteines, if added extracellularly to intact cells with high intracellular reducing environment and for a short period of time (15 s in our study) (Javitch et al., 1994, 2002), the

![Fig. 9. Effects of MTSEA reaction on specific 125I-Tyr3-sauvagine binding to ΔCys, ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, or ΔCys + 364C receptors. Suspensions of HEK 293 cells stably expressing WT CRF1, or ΔCys, ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, or ΔCys + 364C mutants were incubated for 15 s at 22 to 25°C with or without (control) 2.5 mM MTSEA. Thereafter, the cells were homogenized, and membrane homogenates were assayed for specific binding with 125I-Tyr3-sauvagine, as described under Materials and Methods. The bars represent the specific binding (percentage of control), or residual binding, which is defined as the percentage of 125I-Tyr3-sauvagine specific binding to MTSEA-treated receptor divided by the radioligand specific binding to the corresponding untreated receptor. The mean ± S.E. values are from 3 to 4 independent experiments, each performed with duplicate determinations. Asterisk indicates that the residual binding to ΔCys, ΔCys + 211C, ΔCys + 233C, or ΔCys + 364C after MTSEA reaction was not statistically different from the corresponding one to WT (P < 0.05, one-way analysis of variance and least significant difference post hoc test). In contrast, the residual binding to ΔCys, ΔCys + 128C, ΔCys + 211C, ΔCys + 233C, and ΔCys + 364C mutants after MTSEA reaction was not statistically different from each other.

![Fig. 10. Effects of MTSEA reaction on specific 125I-Tyr3-sauvagine binding to ΔCys + 211C + 233C, ΔCys + 211C + 364C, ΔCys + 233C + 364C, or ΔCys + 211C + 233C + 364C receptors. Suspensions of HEK 293 cells stably expressing WT CRF1, or ΔCys, ΔCys + 211C, ΔCys + 211C + 233C, ΔCys + 211C + 364C, ΔCys + 233C + 364C, or ΔCys + 211C + 233C + 364C mutants were incubated for 15 s at 22 to 25°C with or without (control) 2.5 mM MTSEA. Thereafter, the cells were homogenized, and membrane homogenates were assayed for specific binding with 125I-Tyr3-sauvagine, as described under Materials and Methods. The bars represent the specific binding (percentage of control), or residual binding, which is defined as the percentage of 125I-Tyr3-sauvagine specific binding to MTSEA-treated receptor divided by the radioligand specific binding to the corresponding untreated receptor. The mean ± S.E. values are from 3 to 4 independent experiments, each performed with duplicate determinations. Asterisk indicates that the residual binding to ΔCys, ΔCys + 211C, or ΔCys + 233C after MTSEA reaction was significantly different from the corresponding one to WT or to ΔCys + 211C + 233C + 364C (P < 0.05, one-way analysis of variance and least significant difference post hoc test). In contrast, the residual binding to ΔCys, ΔCys + 211C + 233C, ΔCys + 211C + 364C, or ΔCys + 233C + 364C mutants after MTSEA reaction was not statistically different from each other. In addition, the residual binding to WT and to ΔCys + 211C + 233C + 364C after MTSEA reaction was not statistically different from each other.}
endogenous cysteines of CRF₁, which reacted with MTSEA and inhibited 125I-Tyr₀-sauvagine binding, are most likely one or more of the four TM cysteines (Cys128 in the middle of TM1, and Cys211, Cys233, Cys364, which are located near the cytoplasmic ends of TM3, TM4, and TM7, respectively). This suggests that as in family A GPCRs, the TMs of family B GPCRs form a water-accessible crevice with one or more of the endogenous Cys128, Cys211, Cys233, and Cys364 of CRF₁ lying on its surface.

In contrast to MTSEA, MTSET and MTSES did not significantly inhibit 125I-Tyr₀-sauvagine binding to CRF₁. A possible explanation could be that the reactive endogenous cysteines are deep in the crevice such that access of the bulkier MTSET and MTSES is sterically constrained. Likewise, although the cysteines substituted for Ser129 in the cytoplasmic end of TM3 and for Val378 in the cytoplasmic end of TM6 of D2 dopamine receptor were accessible for reaction with MTSET, they did not react with the bulkier MTSET and MTSES (Javitch et al., 1995, 1998). An alternative explanation for the ability of MTSET but not MTSET or MTSES to react is that MTSEA might access the site in its uncharged form, which cannot occur for MTSET or MTSES.

To identify the endogenous cysteines that reacted with MTSEA, we mutated Cys128, Cys211, Cys233, and Cys364, one at a time, to serine, thus creating the mutants C128S, C211S, C233S, and C364S. These mutations did not seem to alter significantly the functional and therefore the structural properties of CRF₁. In contrast to C128S, the mutations, C211S, C233S, and C364S rendered the receptor substantially less sensitive to MTSEA. These results suggest that Cys211 in TM3, Cys233 in TM4, and Cys364 in TM7 are exposed in the water-accessible crevice of receptor and reacted with MTSEA to inhibit 125I-Tyr₀-sauvagine binding. It is also conceivable that these amino acids are located in a water-accessible interface formed by the membrane-spanning domains of two or more different CRF₁ molecules, as observed in other GPCRs (Guo et al., 2008). This possibility is also consistent with the reported ability of CRF₁ to oligomerize (Kraetke et al., 2005b).

Simultaneous mutation to serine of the three reactive cysteines as well as Cys128 and Cys150 greatly decreased the sensitivity to MTSEA of the resulting receptor (ΔCys). There was a small amount of inhibition of ΔCys, despite the fact that the remaining cysteines are believed to be disulfide cross-linked and therefore unreactive (Qi et al., 1997; Pioszak et al., 2008). It is possible that there is partially incomplete disulfide bonding when the receptor is expressed heterologously, which could lead to a small inhibition of sauvagine binding to ΔCys by MTSEA. Another possible explanation could be that MTSEA reacts with a CRF₁-associated protein to allosterically inhibit radioligand binding to CRF₁. Curiously, the amino-terminal extracellular region of CRF₁ receptors, which is essential for radioligand binding, forms a Sushi domain, which has been implicated in protein-protein interactions (Perrin et al., 2006).

Substitution of serine for Cys211, Cys233, or Cys364 rendered the receptor significantly less sensitive to MTSEA. This suggests that no single endogenous cysteine was sufficient, after its reaction with MTSEA, to reduce sauvagine binding to CRF₁. Consistent with this interpretation, reac-
tion of MTSEA with the mutants ΔCys + 211C, ΔCys + 233C, and ΔCys + 364C, which contain only one of the reactive endogenous cysteines, failed to inhibit sauvagine binding. It is noteworthy that simultaneous addition of the three Cys (ΔCys + 211C + 233C + 364C), but no combination of two into ΔCys restored the WT sensitivity to MTSEA. This suggests that MTSEA reacted simultaneously with Cys211, Cys233, and Cys364, to disrupt binding of Tyr₀-sauvagine.

The small nonpeptide CRF₁-selective antagonist antalarmin protected CRF₁ against MTSEA reaction. Antalarmin has been proposed to bind to the TMs of CRF₁ (Liaw et al., 1997; Hoare et al., 2003). Given that the reactive Cys211, Cys233, and Cys364 are expected to be deep within the crevice near the cytoplasmic ends of their TMs, it is likely that antalarmin protected them from MTSEA reaction by binding above them and blocking the passage of reagent from the extracellular medium to the cytoplasmic end of the crevice. It is less likely that this nonpeptide CRF analogue reached the extracellular medium to the cytoplasmic end of TM3, TM4, and TM7 from reaction with MTSEA by binding more extracellularly (Javitch et al., 1995, 1998). These findings are

Fig. 11. Binding affinity of Tyr₀-sauvagine for ΔCys + 211C + 233C, ΔCys + 211C + 364C, ΔCys + 233C + 364C, and ΔCys + 211C + 233C + 364C receptors before and after MTSEA reaction. Suspensions of HEK 293 cells stably expressing WT CRF₁, or the mutants ΔCys, ΔCys + 211C + 233C, ΔCys + 211C + 364C, ΔCys + 233C + 364C, or ΔCys + 211C + 233C + 364C were treated without (control) or with 2.5 mM MTSEA for 15 s at 22 to 25°C. Thereafter, the cells were homogenized and membrane homogenates were incubated with 125I-Tyr₀-sauvagine in the absence or presence of increasing concentrations of Tyr₀-sauvagine. The data were fitted to a one-site competition model by nonlinear regression, and -logKᵦ values were determined as described under Materials and Methods. The bars represent the binding affinity (-logKᵦ) of 125I-Tyr₀-sauvagine before [] or after MTSEA reaction [□]. The mean ± S.E. values are from two to six independent experiments. The results were statistically analyzed using one-way ANOVA followed by least significant difference post hoc test. None of the mutations considerably altered 125I-Tyr₀-sauvagine affinity and MTSEA treatment did not significantly alter radioligand affinity of the receptors tested.
consistent with the inability of the bulkier MTSET to reach the endogenous TM cysteines of CRF1. In addition to the possibility of protection through blocking passage of reagent, we cannot rule out an indirect protection through a ligand-mediated propagated structural rearrangement.

In contrast to our results, theoretical arrangements of the seven helices of family B GPCRs deduced from a detailed analysis of their sequences placed the residues of glucagon-like peptide-1 receptor that correspond to Cys211, Cys233, and Cys364 of CRF1, facing lipid (Donnelly, 1997; Frimurer and Bywater, 1999). Likewise, in the family A GPCRs, D2 dopamine and rhodopsin receptor, residues 4.38 and 4.47, which are predicted to be aligned with Cys211 and Cys233 of CRF1, respectively (Frimurer and Bywater, 1999), face away from the binding site crevice (Baldwin, 1993; Palczewski et al., 2000; Ballesteros et al., 2001). It is conceivable that these cysteines are located at a water-accessible interface of a CRF1 oligomeric complex. However, it is also possible that in a single CRF1 molecule, ligand, local distortions, such as those induced by the presence of proline and/or glycine above Cys211 and Cys233 could alter the configuration of TM3 and TM4 such as to position these cysteines facing into the protein interior, in which they are accessible to MTSEA. Consistent with this proposal, proline and glycine are known to modulate the protein interior. In TM7, the presence of a glycine a few residues above Cys364 might alter the conformation of TM7 to position this Cys facing into the protein interior. Residue 7.54 of the β2-adrenergic and D2 dopamine receptor, which are predicted to be aligned with Cys364 of CRF1, has been shown to be accessible for reaction with MTSEA (Fu et al., 1994). This irregular pattern of accessibility has been proposed to be closely associated with a proline kink at Pro7.50, which is conserved in family A GPCRs, in agreement with the crystal structure of rhodopsin (Fu et al., 1996; Liapakis and Javitch, 1998).

References


cysteines of the corticotropin-releasing factor receptor are critical for ligand interaction. *Biochemistry* 36:12442–12448.

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