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European Journal of Pharmacology 379 (1999) 219–227

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# Expression and characterisation of human and rat $CRF_{2\alpha}$ receptors

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Received 5 July 1999; accepted 9 July 1999

**Abstract**

Rat and human $CRF_{2\alpha}$ receptors were expressed in CHO-pro5 cells and stable cell lines generated. Each receptor was characterised using [$^{125}$I][tyr$^0$]sauvagine and results compared to $CRF_1$ receptors expressed in the same parental cell line. Under identical assay conditions, [$^{125}$I][tyr$^0$]sauvagine labelled both $CRF_1$ and $CRF_{2\alpha}$ receptors with high affinity. The level of expression varied from 103 fmol/mg membrane protein to 1842 fmol/mg membrane protein (rat $CRF_1$ receptors and rat $CRF_2$ receptors, respectively). It was possible to establish robust scintillation proximity assays (SPA) using wheat germ agglutinin (WGA) SPA beads to trap membrane protein. The success of the SPA assay format was dependent on the level of receptor expression observed. The rank order of affinities of a series of peptide CRF receptor agonists and antagonists was similar to that described in the literature for the two receptor subtypes as determined using radioligand binding and cAMP accumulation. No pharmacological differences were apparent between rat and human cloned receptors with the exception of α-helical CRF-(9-41). This peptide exhibited 10-fold higher affinity for rat $CRF_{2\alpha}$ receptors as compared to human $CRF_{2\alpha}$ receptors. PD 173307, PD 173602 and PD 174239 exhibited high affinity and selectivity for human $CRF_1$ receptors, and as such represent useful tools for probing CRF receptor function. 

*Keywords:* $CRF_{2\alpha}$ receptor; [$^{125}$I][tyr$^0$]Sauvagine; CRF receptor antagonist; cAMP

## 1. Introduction

Corticotropin-releasing factor (CRF) is a 41-residue peptide originally isolated from ovine hypothalamus (Vale et al., 1981). Numerous studies have since shown that CRF and related peptides play a major role in stress responses. Thus, CRF has been shown to induce anxiety in rodent and primate models, to modulate food intake (Coskun et al., 1997), blood pressure (Richter and Mulvany, 1995) and thermogenesis (Diamant and De Wied, 1991), and to improve arousal and learning (Heinrichs et al., 1997). These diverse autonomic, electrophysiological and behavioural effects of the CRF family of peptides are mediated through at least two known receptors, $CRF_1$ and $CRF_2$. Both receptors have been previously cloned from various species (Liaw et al., 1996) and the $CRF_2$ receptor type shown to exist as three splice variants of a single gene with different N-terminal domains on the receptor proteins. Distribution studies of the $CRF_2$ receptor have shown that the $CRF_{2\alpha}$ receptor form is located primarily in the brain, and the $CRF_{2\beta}$ receptor form is found predominantly in the periphery in heart and skeletal muscle, with lower levels in the brain, lung and intestine (Lovenberg et al., 1995b). Both splice forms have been described in the rat. The $CRF_{2\beta}$ receptor splice variant, which exhibits $CRF_1$ like receptor pharmacology (Liaw et al., 1996), was initially thought not to occur in human tissues, however a recent study by Valdenaire et al. (1997) has demonstrated a very low level of mRNA expression in human heart and skeletal tissues. The $CRF_{2\gamma}$ receptor isoform has been identified in human brain (Kostich et al., 1998), and exhibits pharmacology similar to that of $CRF_{2\alpha}$ receptor. Numerous studies have reported on the pharmacology of $CRF_1$ receptors, both in vitro and in vivo. The majority of effects described historically may be attributable to this receptor subtype, as the ligands used in these earlier studies have now been shown to be selective for the $CRF_1$ receptor subtype. $CRF_1$ receptors are widely distributed in the central nervous system (CNS) including cortical, cerebellar and sensory relay

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PII: S0014-2999(99)00505-1

structures (Primus et al., 1997). Both $CRF_1$ and $CRF_2$ are G-protein coupled seven trans-membrane protein receptors, with signal transduction occurring via positive coupling to the cAMP pathway.

A number of CRF receptor antagonists have been described in the literature. These include several peptide antagonists, including α-helical CRF-(9-41). This is an N-terminally deleted analogue of the naturally occurring peptides (sauvagine, urotensin I and CRF), which employs the amino acids from each sequence that maximise the α-helical forming potential of the peptide (Rivier et al., 1984). The antagonist properties of this peptide have been shown in a number of bioassays (Fisher et al., 1991). The clinical use of such a compound would be limited due to poor bioavailability and poor ability to permeate the blood–brain barrier. Thus, the lack of suitable high affinity and selective non-peptide antagonists for either $CRF_1$ or $CRF_2$ receptor have hampered progress in this area. The recent development of the high affinity non peptide antagonist CP-154,526 has generated renewed interest in this field. This $CRF_1$ receptor selective compound has been described as a potential anxiolytic in rodents (Schulz et al., 1996).

In this study, we describe the cloning, stable expression and characterisation of human and rat $CRF_2$ receptors expressed in Chinese Hamster Ovary (CHO) pro5 cells. The pharmacological profile of these receptors is compared to $CRF_1$ receptors stably expressed in the same parental cell line, using [$^{125}$I][tyr$^0$]sauvagine in radioligand binding assays. This radioligand has recently been shown to bind with high affinity to both $CRF_1$ and $CRF_2$ receptors (Grigoriadis et al., 1996). Thus, in parallel studies, we have sought to explore interspecies differences in the two receptors, as well as to compare filtration assay format with scintillation proximity techniques. We also describe four new compounds with high affinity and selectivity at the $CRF_1$ receptor (Wustrow et al., 1998).

## 2. Materials and methods

### 2.1. Materials

Oligonucleotide primers were synthesized using an ABI 392 DNA Synthesizer. Human cortex cDNA was obtained from Clontech. The plasmids pBluescript II SK (+) and pcDNA3 were obtained from Stratagene and Invitrogen. *Taq* polymerase was obtained from Perkin-Elmer. Superscript II™ and G418 were obtained from Life Technologies. Restriction enzymes and other DNA modifying enzymes were obtained from Boehringer Mannheim. CHO-Pro5 cells were obtained from American Type Culture Collection (catalogue no CRL-1781). Adult male Wistar rats were obtained from Charles River. [$^{125}$I][tyr$^0$]sauvagine (specific activity 2200 Ci/mmol) was obtained from Dupont NEN, Boston, USA. Peptides were obtained from Bachem (UK), Essex or Sigma, Dorset, UK. Bacitracin and aprotinin and all other chemicals were also purchased from Sigma. All tissue culture media and supplements were obtained from Life Technologies, Gibco. Wheatgerm aggultinin (WGA)-scintillation proximity assay (SPA) beads were obtained from Amersham.

Compounds PD 173307, PD 173602, PD 173713 and PD 174239 (Fig. 1) were synthesised in the Chemistry Department at Parke Davis, Ann Arbor, MI, USA.

### 2.2. Cloning of the human $CRF_{2\alpha}$ receptor

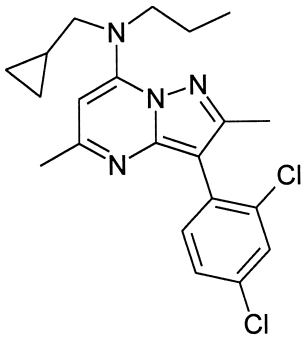
The following oligonucleotide primers were used in polymerase chain reactions (PCR) to amplify human $CRF_{2\alpha}$ receptor sequences. The primers were based on the published human $CRF_{2\alpha}$ receptor cDNA sequence (Genbank accession number U34587, Liaw et al., 1996): HU1 5′ATGGACGCGGCACTGCTCCAC, representing residues 1 to 21; HU2 5′ACCGCATCGCCCTTGTCGTCA, representing residues 338 to 358; HL1 5′GACGCGCGTAACTTTGTCATT, representing the reverse complement of 870 to 890 and HL2 5′CCATCCCACGCCTCCCTCCTC, representing the reverse complement of 1974 to 1994. PCR products were amplified from human cortex cDNA and blunt-end ligated into the *Eco*RV site of pBluescript.

### 2.3. Cloning of the rat $CRF_{2\alpha}$ receptor

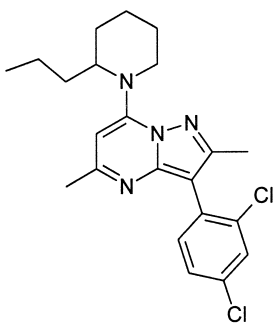
The following oligonucleotide primers were used in PCR reactions to amplify rat $CRF_{2\alpha}$ receptor sequences. The primers were based on the published rat $CRF_{2\alpha}$ receptor cDNA sequence (Genbank accession number U16253, Lovenberg et al., 1995b): RU1 5′TGGGCGGGGAGGCACCTGGAC, representing residues 101 to 121; RU2 5′CGCGCACTCCCACTCCCAACG, representing residues 176 to 196; RL1 5′CCCAAGGGTCAGTGTAGCAAG, representing the reverse complement of 1528 to 1548 and RL2 5′AGGCTGTGAAGAATGAGGAAG, representing the reverse complement of 1494 to 1514. Total RNA was prepared from rat hypothalamus tissue using a standard guanidine thiocyanate method (Sambrook et al., 1989). One microgram RNA was reverse transcribed using Superscript II and an Oligo dT adapter primer. PCR products were amplified from the resulting cDNA and blunt-end ligated into the *Eco*RV site of pBluescript.

### 2.4. Transfection of $CRF_{2\alpha}$ receptors into Chinese hamster ovary cells

Each receptor cDNA was subcloned into the *Hin*dIII–*Eco*RI sites of pcDNA3 (Invitrogen), authenticated by DNA sequencing and used to transfect CHO-Pro5 cells by a modified calcium phosphate precipitation method (based




PD 173307

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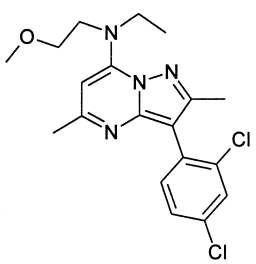


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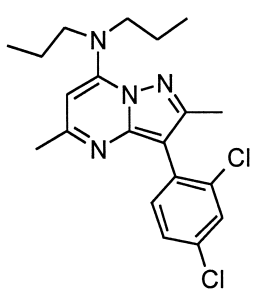


PD 173713

Fig. 1. Structures of PD 173307, PD 173623, PD 174239 and PD 173713.

on Chen and Okayama, 1987). Briefly, 100 mm culture dishes were seeded with $5 \times 10^5$ cells and grown overnight. Twenty micrograms of plasmid DNA was diluted in 0.25 M $CaCl_2$ to 500 μl, mixed with 500 μl 2 × BBS (50 mM BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid), 280 mM NaCl, 1.5 mM $Na_2HPO_4$, pH 6.95) and then added dropwise to a dish of cells. Cells were grown overnight in a 2% $CO_2$ incubator. Cells were then washed and replaced with fresh media and grown for 24 h in a normal 5% $CO_2$ incubator. Cells were subcultured at a 1:10 ratio and grown for a further 24 h before applying selection with G418. G418 resistant cells were diluted and single clones isolated for further culture and evaluation.

Cloning and generation of stable cell lines expressing rat and human $CRF_1$ receptors was performed at the Parke Davis laboratories in Michigan, USA (Pugsley and MacKenzie, personal communication).

### 2.5. Cell culture

CHO-Pro5 cells expressing either human or rat $CRF_1$ or $CRF_2$ receptors were grown as monolayers in minimum essential medium (MEM)-alpha, supplemented with 10% foetal calf serum under 5% $CO_2$ at 37°C. The cells were passaged every 4–5 days, by re-seeding at a density of 1–2 million per 175 $cm^2$ flask. Cells were passaged for up to fifteen times before reseeding from frozen stocks.

### 2.6. Preparation of membranes

Cells were harvested 4 or 5 days after passaging by scraping into PBS/1 mM EDTA, followed by centrifugation at $1500 \times g$ for 4 min in a Beckman GPR centrifuge. The resulting pellet was resuspended in preparation buffer (Tris–HCl, pH 7.2 at 4°C, containing 2 mM EGTA, 5 mM $MgCl_2$, 80 μg/ml bacitracin and 150 kIU/ml aprotinin), using a Brinkman polytron (setting 5 for 10 s) and washed by centrifugation at $3500 \times g$ for 10 min. The pellet was resuspended in preparation buffer as before and stored frozen in 1 ml aliquots at −70°C until required for use in binding assays. Protein content of the cell preparation was determined by the method of Lowry et al. (1951).

### 2.7. Radioligand binding assays

For competition studies, CHO-Pro5 cell membranes were incubated with [$^{125}$I][tyr$^0$]sauvagine (40–120 pM) in

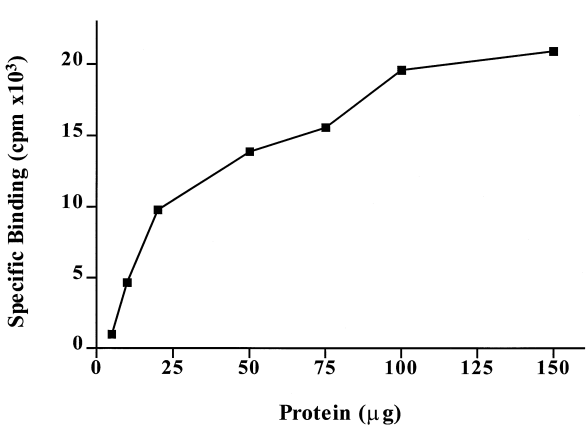


Fig. 2. Effect of increasing membrane protein concentration on specific binding of [$^{125}$I][tyr$^0$]sauvagine, using rat $CRF_{2\alpha}$ receptors stably expressed in CHO-pro5 cells. The graph shows data from a single representative experiment performed in duplicate, as described in Section 2.

the presence or absence of test compounds for 120 min at room temperature in assay buffer (Tris–HCl, pH 7.2 at 22°C, containing 2 mM EGTA, 5 mM $MgCl_2$, 80 μg/ml bacitracin,150 kIU/ml aprotinin and 0.15% bovine serum albumin) in a total volume of 250 μl. Protein concentrations of 150, 10, 30 and 100 μg per tube were used for human $CRF_{2\alpha}$, rat $CRF_{2\alpha}$, human $CRF_1$ and rat $CRF_1$, respectively. 5 μM rat/human CRF was used to define non-specific binding. Assays were terminated by vacuum filtration (using a Brandel cell harvester) over GF/C filters presoaked in 0.15% bovine serum albumin/10 mM HEPES and the filters were washed 5 times with 1 ml Tris–HCl (50 mM, pH 6.9 at 22°C). Radioactivity bound in counts per minute (cpm) was determined using a gamma counter.

For saturation studies, specific binding of [$^{125}$I][tyr$^0$]sauvagine was determined over a range of radiolabel concentrations (10–2000 pM). For kinetic studies, membranes were incubated with [$^{125}$I][tyr$^0$]sauvagine in the presence or absence of 5 μM rat/human CRF and aliquots taken at various time points and filtered using a Millipore manifold.

Scintillation proximity assays were carried out under buffer conditions identical to those used in filtration assays. Conditions were optimised with respect to bead and cell membrane concentration prior to performing characterisation assays. Thus, all experiments were performed using 0.625 mg beads per well and 75 μg per well or 300 μg per well of rat $CRF_2$ and human $CRF_1$ membrane protein respectively, in a total volume of 250 μl. Specific binding was determined using a Packard TopCount.

### 2.8. Analysis of intracellular cyclic AMP levels

For measurement of cyclic AMP levels, cells were harvested, centrifuged at $200 \times g$ for 2 min and resuspended in cyclic AMP assay buffer (120 mM NaCl, 5.4 mM KCl, 1.6 mM $MgCl_2$, 25 mM HEPES, 1.2 mM $KH_2PO_4$, pH 7.4 with 11 mM glucose, 1.8 mM $CaCl_2$ and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX)) pre-warmed to 37°C and gassed with $O_2$. Cells were counted and diluted to $\sim 6 \times 10^5$ cells/ml with assay buffer. Cell aliquots (50 μl) were added to eppendorf tubes and treated for 10 min at 37°C with assay buffer containing the appropriate agent. The reaction was stopped by the addition of 100 μl ice cold perchloric acid containing 1 mM EDTA and the samples incubated at 4°C for between 15–60 min. Samples were centrifuged at $2000 \times g$ for 5 min at 4°C and the resulting supernatants transferred to fresh tubes. Samples were extracted with 400 μl 1:1 trichloro-trifluoroethane:tri-*n*-octylamine by vortexing and then centrifuged at 4°C for 5 min at $2800 \times g$. A 50-μl aliquot of the upper phase was removed from each tube and assayed for cAMP using the Amersham cAMP [$^3$H] Biotrak radioreceptor assay system according to the manufacturer's instructions.

### 2.9. Data analysis

Nonlinear regression analyses of saturation, kinetic and competition assay data was performed with iterative curve-fitting procedures in GRAPHPAD Prism. All $IC_{50}$ values were converted to $K_i$ values using the Cheng–Prusoff equation (Cheng and Prussoff, 1973).

## 3. Results

### 3.1. Cloning of the human $CRF_{2\alpha}$ receptor

A clone representing the full-length cDNA for the human $CRF_{2\alpha}$ receptor was constructed from two separate PCR products. Many different combinations of oligonucleotide primers (not shown) were used in multiple rounds of PCR to try to amplify the human $CRF_2$ receptor from cortex cDNA. The largest of these products were obtained in separate reactions after two rounds of PCR using primers HU1 with HL1 and HU2 with HL2. These were purified and subcloned into pBluescript for sequence analysis. The two clones obtained represented the first 72% and the last 75% of the coding region. A clone representing the full coding region was obtained by restriction endonuclease digestion of each clone at a common

Table 1
Comparison of $K_D$ and $B_{max}$ values for binding of [$^{125}$I][tyr$^0$]sauvagine to rat and human $CRF_1$ and $CRF_{2\alpha}$ receptors expressed in CHO-pro5 cells using standard filtration and scintillation proximity assay formats. Saturation studies were performed in duplicate as described in Section 2. The data shown represent mean values ± S.E.M. determined in 3–5 separate experiments
ND: not determined.

| | Filter assays | | SPA | |
|---|---|---|---|---|
| | $B_{max}$ (fmol/mg) | $K_D$ (nM) | $B_{max}$ (fmol/mg) | $K_D$ (nM) |
| $CRF_1$ human | 537 ± 35 | 2.442 ± 0.15 | ND | ND |
| $CRF_1$ rat | 103 ± 21 | 0.536 ± 0.17 | ND | ND |
| $CRF_{2\alpha}$ human | 56.2 ± 30 | 0.274 ± 0.04 | ND | ND |
| $CRF_{2\alpha}$ rat | 1842 ± 34 | 0.403 ± 0.02 | 1569 ± 535 | 1.3242 ± 0.244 |

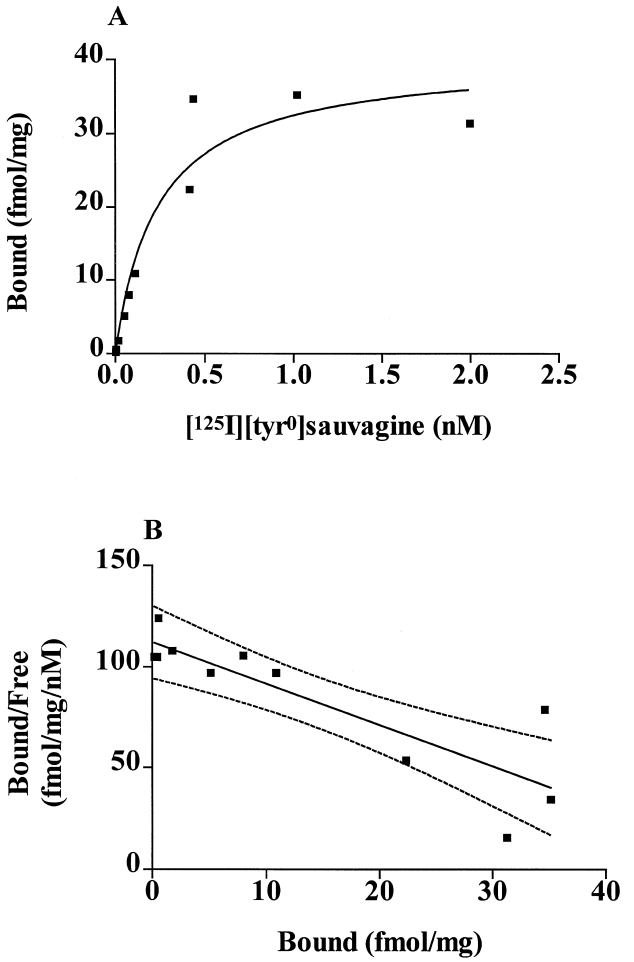


Fig. 3. (A) Saturation of [$^{125}$I][tyr$^0$]sauvagine binding to membranes prepared from CHO-pro5 cells expressing cloned human $CRF_{2\alpha}$ receptors. The graph shows data from a single representative experiment performed in duplicate as described in Section 2. $K_D$ and $B_{max}$ values shown in Table 1 are the means taken from three such experiments. (B) Scatchard transformation indicating binding to a single population of sites.

internal *Bam*HI site and ligation of the two parts. The resultant full-length clone was identical to the published sequence with only four point mutations, indicating no alternatively spliced variants. The four point mutations obtained were due to the infidelity of *Taq* polymerase as confirmed by further PCR investigation. These changes were corrected by site-directed mutagenesis and the majority of the 3″ untranslated region was removed by the introduction of an *Eco*RI site 28 bases after the termination codon to obtain the original published sequence.

### 3.2. Cloning of the rat $CRF_{2\alpha}$ receptor

A clone representing a full coding region cDNA for the rat $CRF_{2\alpha}$ receptor was obtained using a nested PCR protocol. The first PCR reaction used a combination of primers RU1 and RL1 with rat hypothalamus cDNA. The second PCR reaction used the products from the first reaction with a combination of primers RU2 and RL2. The product was purified and blunt-end ligated into pBluescript for sequence analysis. The resultant clone was almost identical to the published sequence with only four point mutations again due to mis-incorporation by *Taq* polymerase. These changes were corrected by site-direct mutagenesis to obtain the original published sequence before the cDNA was ligated into pcDNA3.

### 3.3. Generation of stable cells lines

Thirty six and 39 populations of rat and human cells derived from single cells respectively were established and evaluated for ability to bind [$^{125}$I][tyr$^0$]sauvagine. Clonal lines exhibited a range of expression levels with differing degrees of specific binding. Two cell lines (one rat and one human), which maintained a consistent level of expression over at least 5 passages and exhibited functional responses

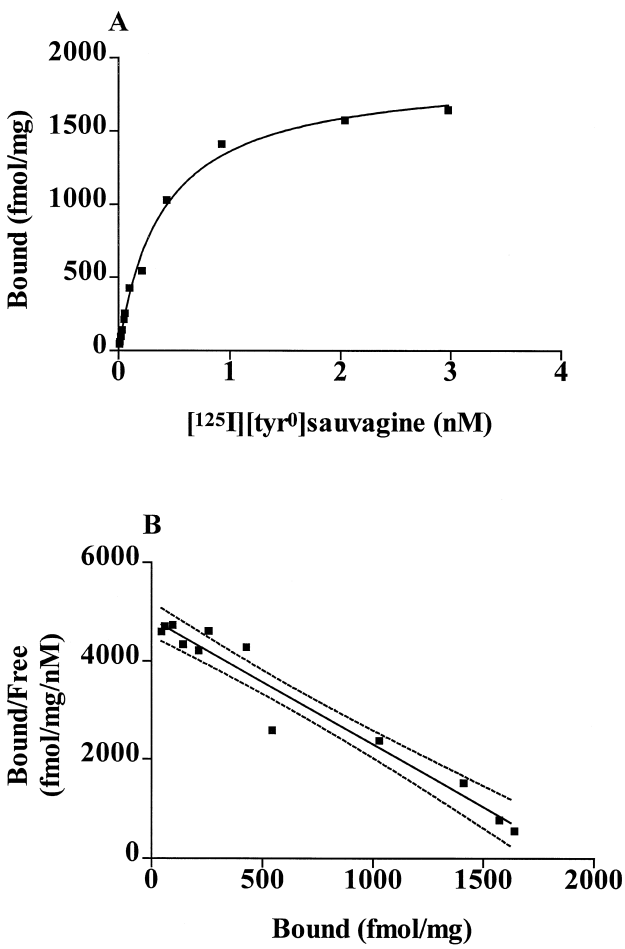


Fig. 4. (A) Saturation of [$^{125}$I][tyr$^0$]sauvagine binding to membranes prepared from CHO-pro5 cells expressing cloned rat $CRF_{2\alpha}$ receptors. The graph shows data from a single representative experiment performed in duplicate as described in the Section 2. $K_D$ and $B_{max}$ values shown in Table 1 are the means taken from at least three such experiments. (B) Scatchard transformation indicating binding to a single population of sites.

Table 2
Affinities of CRF receptor agonists and antagonists for [$^{125}$I][tyr$^0$]sauvagine binding to human and rat $CRF_1$ and $CRF_{2\alpha}$ receptors. Experiments were performed in duplicate as described in Section 2. The data shown represents the geometric mean and range of $K_i$ values determined in 3–10 separate experiments

| Compound | $K_i$ (nM) (range) | | | |
|---|---|---|---|---|
| | Human $CRF_1$ | Rat $CRF_1$ | Human $CRF_{2\alpha}$ | Rat $CRF_{2\alpha}$ |
| Rat/human CRF | 3.7 (1.7, 5.6) | 1.0 (0.4, 2.7) | 140 (93, 210) | 35(10, 63) |
| Ovine CRF | – | 1.2 (1.1, 1.4) | – | 152(117, 201) |
| Sauvagine | 0.56 (0.1, 2.1) | 0.8 (0.5, 1.5) | 3.4 (2.0, 18) | 1.2 (0.3, 3.3) |
| Urotensin I | 0.75 (0.5, 1.7) | 0.9 (0.7, 1.2) | 36 (18, 91) | 11 (10, 11) |
| Urotensin II | > 10,000 | > 10,000 | > 10,000 | > 10,000 |
| Rat urocortin | 0.7 (0.5, 1.3) | 0.6 (0.3, 2.2) | 1.1 (0.8, 1.5) | 2.4 (1.3, 4.5) |
| Human urocortin | 0.6 (0.3, 2.4) | 0.8 (0.4, 1.6) | 1.5 (0.5, 3.4) | 2.3 (0.5, 7.0) |
| Astressin | 2.4 (1.4, 5.9) | 0.4 (0.4, 0.5) | 1.6 (0.8, 3.7) | 0.9 (0.2, 2.0) |
| α-helical CRF-(9-41) antagonist | 156 (64, 500) | 17 (5, 48) | 360 (88, 570) | 25 (6, 95) |
| [(D-Phe$^{12}$,Nle$^{21,38}$,α-Me-Leu$^{37}$)] CRF-(12-41) (rat/human) | 35 (19, 98) | 36 (24, 55) | 20 (7.5, 48) | 9.5 (5.6, 25) |
| PD173307 | 29 (10, 67) | 29 (13, 46) | > 1000 | > 1000 |
| PD173602 | 139 (98, 246) | 32 (12, 140) | > 1000 | > 1000 |
| PD173713 | > 1000 | > 1000 | > 1000 | > 1000 |
| PD174239 | 35 (14, 100) | 15 (7.8, 33) | > 1000 | > 1000 |

to activation of CRF receptors, were selected for detailed characterisation.

### 3.4. Radioligand binding studies

Maximum receptor expression in CHO-pro5 cells was seen 4-5 days after passaging (data not shown). Under the conditions described, [$^{125}$I][tyr$^0$]sauvagine bound to both $CRF_1$ and $CRF_2$ rat and human receptors with high affinity. Specific binding, as defined by the presence of 5 μM rat/human CRF, was in the range 50–70 and 75–90%, for human and rat $CRF_2$ receptors respectively, and increased with increasing membrane concentration over the range 20–250 μg/tube and 10–100 μg/tube for human and rat $CRF_{2\alpha}$ receptors (Fig. 2), respectively. Specific binding of > 80% was routinely obtained for both rat and human $CRF_1$ receptors.

Saturation studies indicated that [$^{125}$I][tyr$^0$]sauvagine bound with high affinity to all four receptor types, with $K_D$ values in the range 0.28–2.4 nM (Table 1). Scatchard analysis yielded linear plots indicating binding to a single non-interacting site in each case (Figs. 3 and 4). Maximum binding capacity varied markedly between the various receptors, with the $CRF_2$ human clonal line exhibiting the lowest level and the $CRF_2$ rat clonal line exhibiting the highest level of expression (Table 1).

Specific binding of [$^{125}$I][tyr$^0$]sauvagine was inhibited in a concentration dependent manner by a variety of CRF receptor ligands (Table 2; Fig. 5). The rank order of affinity at human $CRF_1$ receptors and human $CRF_2$ receptors were: Sauvagine = Astressin = urocortin = rat/human CRF = ovine CRF = urotensin I > (D-Phe$^{12}$,Nle$^{21,38}$,α-Me-Leu$^{37}$) CRF-(12-41) ≥ α-helical CRF-(9-41) > Urotensin II, and sauvagine = Astressin = urocortin > (D-Phe$^{12}$, Nle$^{21,38}$,α-Me-Leu$^{37}$) CRF-(9-41) = Urotensin I > rat/human CRF > α-helical CRF-(9-41) > Urotensin II, re-

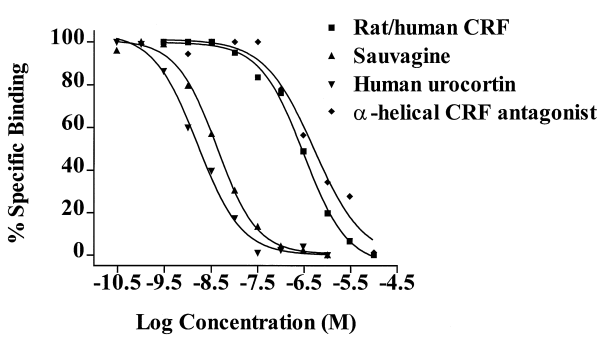


Fig. 5. Inhibition of specific [$^{125}$I][tyr$^0$]sauvagine binding to membranes prepared from CHO-pro5 cells expressing human $CRF_2$ receptors using filtration assay format. Results shown in Table 2 represent the geometric means taken from at least three such experiments.

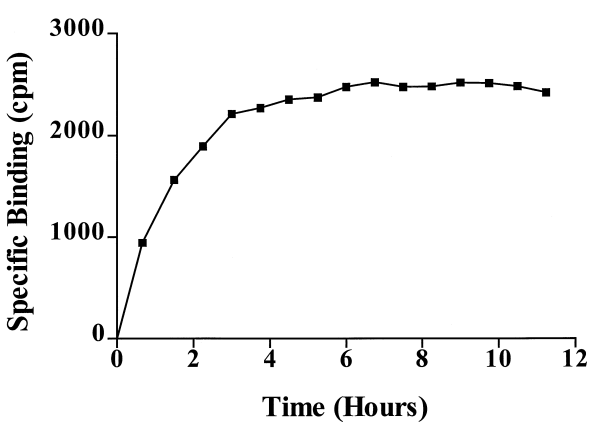


Fig. 6. Association of [$^{125}$I][tyr$^0$]sauvagine binding to $CRF_2$ rat receptors stably expressed in CHO cells using scintillation proximity assay technology. The graph shows data from a single representative experiment performed in triplicate, as described in Section 2.

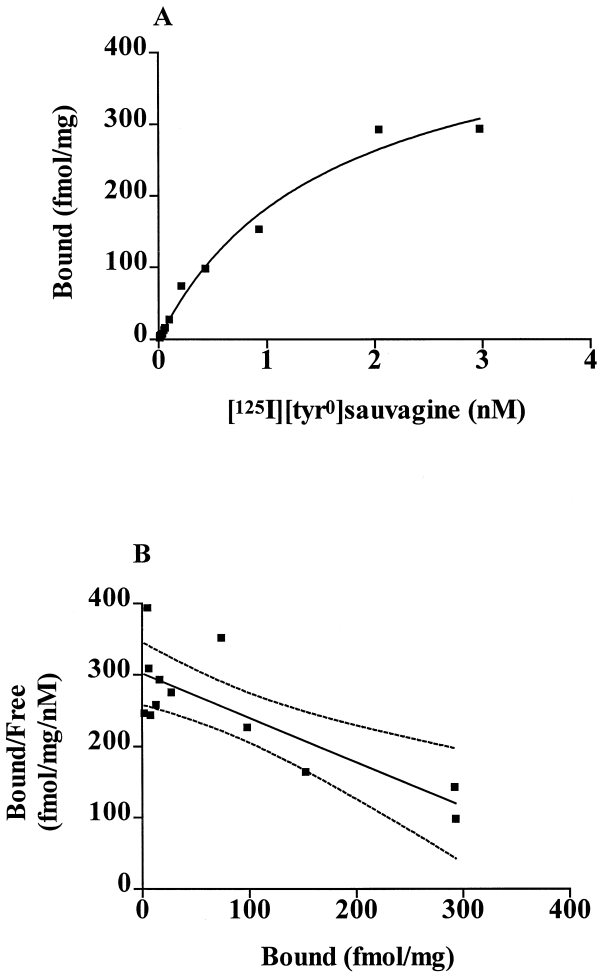


Fig. 7. Saturation of [$^{125}$I][tyr$^0$]sauvagine binding to membranes prepared from CHO-pro5 cells expressing cloned rat $CRF_{2\alpha}$ receptors performed using scintillation proximity assay format. The graph shows data obtained from a single representative experiment performed in duplicate as described in Section 2. $K_D$ and $B_{max}$ values shown in Table 1 are the means taken from at least three such experiments.

spectively. The rank order of affinities obtained for the respective rat receptors was similar to those obtained in the human receptor assays, with the exception of α-helical CRF-(9-41). In our hands, this peptide antagonist exhibited over 10 fold higher affinity for rat $CRF_{2\alpha}$ receptors as compared to human $CRF_{2\alpha}$ receptors. PD 173307, PD 173602, and PD 174239 exhibited $K_i$ values in the range 15–140 nM and $>1000$ nM at $CRF_1$ and $CRF_{2\alpha}$ receptors respectively (Table 2). PD 173713 was inactive at both receptors.

Where it was possible to establish a robust homogeneous assay, scintillation proximity assays were performed in parallel with filtration assays in order to validate this technology for peptide receptors. A steady state was reached 5 h after initiation of association by mixing membrane-coated beads with radioligand (Fig. 6). This was maintained for up to 14 h. Saturation analyses of rat $CRF_{2\alpha}$ receptors using the SPA format yielded similar results to filtration assays (Table 1; Fig. 4; Fig. 7).

In general, the rank order of potency was similar to that obtained in filtration assays (Table 3), although the absolute affinities were 2–3-fold lower. Again, the only exception was α-helical CRF-(9-41), which exhibited markedly lower affinity at the rat $CRF2_{\alpha}$ receptor when evaluated in SPA format.

### 3.5. Cyclic AMP stimulation studies

Functional activity of $CRF_{2\alpha}$ receptors was monitored by evaluation of CRF receptor-mediated stimulation of adenylyl cyclase activity. The effects of sauvagine, rat/human CRF and ovine CRF on intracellular cyclic AMP levels were compared in cells expressing $CRF_1$ and $CRF_{2\alpha}$ receptors stimulated with agonist for a total of 10 min. In three independent experiments, stimulation of $CRF_1$ receptor expressing cultures with CRF receptor agonists revealed dose-dependent increases in intracellular cyclic AMP concentration above basal values (Table 4). Stimulation of cultures expressing $CRF_{2\alpha}$ receptors with CRF receptor agonists also revealed a dose-dependent increase in intracellular cyclic AMP concentrations. These provided

Table 3
Affinities of CRF receptor agonists and antagonists for [$^{125}$I][tyr$^0$]sauvagine binding to human and rat $CRF_1$ and $CRF_{2\alpha}$ receptors using scintillation proximity assay format. Experiments were performed in duplicate as described in Section 2. The data shown represent the geometric means of $K_i$ or $IC_{50}$ values determined in 3–6 separate experiments

| Compound | $IC_{50}$ (nM) (range) human $CRF_1$ | $K_i$ (nM) (range) Rat $CRF_{2\alpha}$ |
|---|---|---|
| Rat/human CRF | 3.5 (2.1, 7.4) | 410 (238, 645) |
| Sauvagine | 0.6 (0.2, 1.0) | 4.1 (2.1, 11) |
| Urotensin I | 0.8 (0.2, 1.5)[a] | 25 (13, 45) |
| Urotensin II | >10,000 | >10,000 |
| Rat Urocortin | 0.3 (0.2, 0.4) | 1.2 (0.6, 2.0) |
| Human Urocortin | 0.3 (0.03, 0.7) | 1.1 (0.8, 1.3) |
| Astressin | 11 (6.3, 14) | 8.7 (1.7, 56) |
| α-helical CRF-(9-41) antagonist | 12 (9.0, 14)[a,b] | 810 (560, 1220) |
| [(D-Phe$^{12}$,Nle$^{21,38}$,α-Me-Leu$^{37}$)] CRF-(12-41) (rat/human) | 154 (115, 327) | 32 (22, 41) |

[a] $n = 2$.
[b] Variable data. Further repeated testing resulted in $IC_{50}$ values $>1$ μM.

Table 4
CRF receptor agonist induced cyclic AMP stimulation in CHO-pro5 cells expressing rat $CRF_1$ and $CRF_{2\alpha}$ receptors. The data shown represents the geometric mean and range of $EC_{50}$ values determined in at least 3 separate experiments

| Ligand | $CRF_1$ | | $CRF_{2\alpha}$ | |
|---|---|---|---|---|
| | $EC_{50}$ (nM) | Range | $EC_{50}$ (nM) | Range |
| Sauvagine | 1.3 | (0.7, 2.8) | 0.2 | (0.14, 0.23) |
| Rat/human CRF | 1.5 | (1.4, 1.6) | 2.8 | (1.7, 4.6) |
| Ovine CRF | 1.8 | (1.4, 2.9) | 33 | (26, 43) |

a rank order of potency of sauvagine = rat/human CRF = ovine CRF ($CRF_1$ cells) and sauvagine > rat/human CRF > ovine CRF ($CRF_{2\alpha}$ cells). Sauvagine was without effect on intracellular levels of cyclic AMP in the CHO-pro5 parental cell line demonstrating the specificity of the cyclic AMP response (data not shown).

## 4. Discussion

Modulation of CRF as a target is of particular interest to the pharmaceutical sector, due to involvement of CRF in a wide range of peripheral and central functions. In particular, alterations in CRF content have been shown to occur in a variety of CNS disorders. Thus, CRF levels have been reported to be decreased in Alzheimers patients (De Souza et al., 1986), and increased in suicide victims and depressed patients (for review see Nemeroff, 1992). CRF levels may also be important in other psychiatric disorders, e.g., schizophrenia (Owens and Nemeroff, 1993). Further more, urocortin, the putative endogenous mammalian ligand for $CRF_2$ receptors, has been shown to be anxiogenic in several rodent behavioural paradigms (Moreau et al., 1997), thus implicating a role for $CRF_2$ receptors in anxiety. Such speculation led Meyer et al. (1997) to identify the $CRF_2$ receptor on human chromosome 7p21-p15, using fluorescence in situ hybridisation and radiation hybrid mapping techniques. This location does not appear close to the currently known hot spots of genes associated with schizophrenia and bipolar depression. Further correlations with other anxiety or depressive disorder gene hot spots may aid identification of the role of the $CRF_2$ receptor.

$CRF_1$ and $CRF_2$ receptors have been cloned by a number of laboratories from rat, mouse and human sources. In the present study we have cloned, expressed and characterised cloned rat and human $CRF_1$ and $CRF_{2\alpha}$ receptors stably expressed in CHO-pro5 cells using [$^{125}$I][tyr$^0$]-sauvagine to radiolabel both receptors. The majority of such studies in the past have utilised iodinated ovine CRF. This radioligand does not label $CRF_2$ receptors, and until recently alternative high affinity radioligands were not available for labelling the $CRF_2$ receptor. The availability of radiolabelled sauvagine, which retains high affinity for the $CRF_2$ as well as $CRF_1$ receptor, has enabled high affinity labelling of both receptors, allowing comparison of affinities of compounds under identical conditions.

The pharmacological profile of both receptor types in this study was in agreement with published data. In addition, the data obtained in these experiments indicate that there is no interspecies difference in the pharmacological profile of rat and human $CRF_1$ and $CRF_2$ receptors. The only discrepancy observed is with α-helical CRF-(9-41). This compound has been shown to be a partial agonist in some in vitro and in vivo systems (Fisher et al., 1991), and marked variation has been shown to occur with respect to doses required in different assay systems. Therefore it is not possible to interpret the data obtained in this study as a species difference.

A number of antagonists have been described in the literature. In this study, we present data on three compounds with high affinity and selectivity for the $CRF_1$ receptor (Table 2). All are antagonists at $CRF_1$ receptors (Smart et al., 1999, = 13 124). Such tools will be useful in the study of CRF receptors. In particular, the development of similar selective tools for $CRF_2$ receptors will facilitate research and define the roles of the different receptor subtypes in various stress states.

This study also attempted to compare a scintillation proximity assay (Udenfriend et al., 1987) format to standard filtration format. SPA is now a well established homogeneous screening technology enabling higher throughput of compounds. Scintillation proximity formats have been described for a variety of receptors, including some peptide receptors, in the application notes published by Amersham. In our hands, it was only possible to establish robust assays (specific binding > 1000 cpm at a steady state) for two of the four cloned receptors characterised in this study (Tables 1 and 3). This appears to correlate with the level of expression, with cells exhibiting highest receptor expression generating the highest specific binding. In general the pharmacological profiles were similar to those obtained using the filtration assay format. Thus, high affinity compounds exhibited approximately equal affinity in the two assay formats, with more variation being observed for the lower affinity compounds. It was not possible to define the reason for the discrepancies observed between the two assay formats for α-helical CRF-(9-41) antagonist, and the degree of variation observed in $IC_{50}$ values for this compound at the human $CRF_1$ receptor. One possibility was that the concentration of antagonist in the assay may have been overestimated due to either a solubility issue, or to losses by adsorption onto the surface of the SPA beads or the microtitre plate in which the assay is performed. We attempted to eliminate the latter possibility by performing the assay in the presence of different concentrations of bovine serum albumin to saturate potential adsorption sites; however, no differences in affinity were observed for either the antagonist, or any other peptide evaluated in the SPA format (unpublished).

Data from functional studies measuring CRF receptor-mediated stimulation of cyclic AMP (Table 4) confirmed the profile of activity obtained in receptor binding studies. Sauvagine was without effect on intracellular levels of cyclic AMP in the CHO-pro5 parental cell line confirming the specificity of the receptor-mediated cyclic AMP response. These data are in broad agreement with those obtained by analysis of cyclic AMP production in a variety of cell lines including CHO cells stably expressing cloned human $CRF_1$ and $CRF_{2\alpha}$ receptors, (Donaldson et al., 1996; Grigoriadis et al., 1996), COS-M6 cells expressing rat $CRF_1$ receptors (Vaughan et al., 1995) and $LtK^-$ cells overexpressing rat $CRF_1$ and rat $CRF_{2\alpha}$ receptors (Lovenberg et al., 1995a). Together these data confirm that $CRF_1$ and $CRF_{2\alpha}$ receptors modulate cyclic AMP signalling pathways and reinforce the validity of the stable cell lines such as those generated in the current study as model systems for the study of CRF-mediated signalling events.

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